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04/07/99

TO: Assistant Commissioner for Patents
Box Patent Applications
Washington D.C. 20231

Attorney Docket No. **16777/309**
(must include alphanumeric codes if no inventors named)

UTILITY PATENT APPLICATION TRANSMITTAL
(new nonprovisional applications under 37 CFR 1.53(b))

Transmitted herewith for filing is the patent application of:

INVENTOR(S): Gautvik et al.

TITLE: PRODUCTION OF HUMAN PARATHYROID HORMONE FROM MICROORGANISMS

In connection with this application, the following are enclosed:

APPLICATION ELEMENTS:

☒ Specification - 48 TOTAL PAGES

(preferred arrangement:)

- Descriptive Title of the Invention
- Cross Reference to Related Applications
- Statement Regard Fed sponsored R&D
- Reference to Microfiche Appendix
- Background of the Invention
- Brief Summary of the Invention
- Brief Description of the Drawings (if filed)
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

☒ Drawings - Total Sheets 17

☒ Declaration and Power of Attorney - Total Sheets 2

☐ Newly executed (original or copy)

☒ Copy from a prior application (37 CFR 1.63(d))

(relates to continuation/divisional boxes completed) - NOTE: Box below

☐ DELETION OF INVENTOR(S) - Signed statement attached deleting inventor(s)
named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).

☒ Incorporation By Reference (useable if copy of prior application
Declaration being submitted)

The entire disclosure of the prior application, from which a COPY of the oath or declaration is supplied as noted above, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.

☐ Microfiche Computer Program (Appendix)

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- ☐ Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary)
- ☐ Computer Readable Copy
 - ☐ Paper Copy (identical to computer copy)
 - ☐ Statement verifying identify of above copies

ACCOMPANYING APPLICATION PARTS

- ☒ Assignment Papers (cover sheet & document(s))
- ☐ 37 CFR 3.73(b) Statement (when there is an assignee)
- ☐ English Translation Document (if applicable)
- ☐ Information Disclosure Statement(IDS) with PTO-1449. ☐ Copies of IDS Citations
- ☒ Preliminary Amendment
- ☒ Return Receipt Postcard (MPEP 503)
- ☐ Small Entity Statement(s)
- ☐ Statement file in prior application, status still proper and desired.
- ☐ Certified Copy of Priority Document(s) with Claim of Priority
(if foreign priority is claimed).

OTHER:

If a **CONTINUING APPLICATION**, check appropriate box and supply the requisite information:

- ☒ Continuation ☐ Divisional ☐ Continuation-in-part (CIP)
of prior application Serial No. ☐.

☒ Amend the specification by inserting before the first line the following sentence: --This application is a ☐ continuation of application Serial No. 08/340,664, filed November 16, 1994.--

CORRESPONDENCE ADDRESS:

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FEE CALCULATIONS: (Small entity fees indicated in parentheses.)

(1) For	(2) Number Filed	(3) Number Extra	(4) Rate	(5) Basic Fee \$760 (\$380)
Total Claims	17 - 20 =	0	x \$18 (x \$ 9)	\$0.00
Independent Claims	6 - 3 =	3	x \$78 (x \$39)	\$234.00
Multiple Dependent Claims			\$260 (\$130)	\$0.00
Assignment Recording Fee per property			\$40	\$0.00
Surcharge Under 37 C.F.R. 1.16(e)			\$130 (\$65)	\$0.00
TOTAL FEE:				\$994.00

METHOD OF PAYMENT:

A check in the amount of \$994.00 is enclosed.

If payment by check is NOT enclosed, it is requested that the Patent and Trademark Office advise the undersigned of the period of time within which to file the TOTAL FEE. If payment is enclosed, this amount is believed to be correct; however, the Commissioner is hereby authorized to charge any deficiency or credit any overpayment to Deposit Account No. 19-0741.

Respectfully submitted,

Date: April 7, 1999
Docket No. **16777/309**

Michele M Schafer
Michele M. Schafer
Registration No. 34,717

[illegible]

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:)
)
Gautvik *et al.*)
)
Continuation of Serial No.: 08/340,664) **Prior Application:**
) Group Art Unit: 1646
)
Filed: Herewith) Examiner: L. Spector
)
For: PRODUCTION OF HUMAN PARA-)
THYROID HORMONE FROM)
MICROORGANISMS)

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as follows.

IN THE CLAIMS:

Please cancel claims 1-20, without disclaimer or prejudice thereof, and add the following claims.

21. (Amended) A composition comprising recombinant human parathyroid hormone (hPTH) (1-84), [as a substantially homogeneous protein] wherein:

(a) the hormone is resistant to degradation; and

(b) the hormone has a point mutation at amino acid 26 changing the amino acid from lysine (K26) to glutamine (Q26).

22. The composition of claim 21, wherein the composition has a hPTH (1-84) purity of at least 80%.

23. The composition of claim 22, wherein the hPTH (1-84) is nearly homogeneous.

24. A composition comprising recombinant human parathyroid hormone (hPTH) (1-84), wherein the hPTH is made by a process comprising the steps of:

- (a) providing a microorganism comprising:
 - (1) a leader sequence corresponding to the DNA sequence encoding *Saccharomyces* mating factor $\alpha 1$ lacking the yeast STE13 recognition; and
 - (2) a DNA sequence encoding hPTH, wherein the leader sequence and the hPTH sequence are operably linked;
- (b) culturing said microorganism to allow expression of said DNA sequence encoding hPTH, thereby producing hPTH (1-84); and
- (c) purifying the resultant hPTH (1-84) protein.

25. The composition of claim 24, wherein the microorganism is selected from the group consisting of *Escherichia coli* and yeast.

26. The composition of claim 24, wherein the hPTH protein has a purity of greater than 90%.

27. A composition comprising recombinant human parathyroid hormone (hPTH) (1-84), wherein the hPTH is made by a process comprising the steps of:

- (a) providing a microorganism comprising:
 - (1) the first nineteen amino acids of the DNA sequence encoding *Saccharomyces* mating factor $\alpha 1$ as a leader sequence; and
 - (2) a DNA sequence encoding hPTH, wherein the leader sequence and the hPTH sequence are operably linked;
- (b) culturing said microorganism to allow expression of said DNA sequence encoding hPTH, thereby producing hPTH (1-84); and

- (c) purifying the resultant hPTH (1-84) protein.

28. The composition of claim 27, wherein the microorganism is selected from the group consisting of *Escherichia coli* and yeast.

29. The composition of claim 27, wherein the protein has a purity of greater than 90%.

30. A composition comprising recombinant human parathyroid hormone (hPTH) (1-84), wherein the hPTH is made by a process comprising the steps of:

- (a) providing a microorganism comprising:
- (1) a leader sequence; and
 - (2) a DNA sequence encoding a derivative of hPTH, wherein the cleavage site after the pair of basic amino acids at positions 25 and 26 of the derivative hPTH gene has been modified such that the hormone is excluded as a substrate for yscF protease, wherein the leader sequence and the hPTH sequence are operably linked;
- (b) culturing said microorganism to allow expression of said DNA sequence encoding hPTH, thereby producing hPTH (1-84); and
- (c) purifying the resultant hPTH (1-84) protein.

31. The composition of claim 30, wherein amino acid 26 of the human hPTH gene is modified from lysine to glutamine.

32. The composition of claim 30, wherein the leader sequence is the DNA sequence encoding *Saccharomyces* mating factor $\alpha 1$.

33. The composition of claim 30, wherein the protein has a purity of greater than 90%.

34. A composition comprising recombinant parathyroid hormone (hPTH) (1-84), wherein the hPTH is made by a process comprising the steps of:

- (a) providing a microorganism comprising:
 - (1) a leader sequence; and
 - (2) a DNA sequence encoding hPTH comprising an optimized consensus signal sequence having the following:
 - (i) a positively charged amino-terminal;
 - (ii) a hydrophobic core region; and
 - (iii) a polar COOH-terminal region,wherein the leader sequence and the hPTH sequence are operably linked;
- (b) culturing said microorganism to allow expression of said DNA sequence encoding hPTH, thereby producing hPTH (1-84); and
- (c) purifying the resultant hPTH (1-84) protein.

35. The composition of claim 34, wherein the signal sequence is encoded by an amino acid sequence selected from the group consisting of: (1) Met-Lys-Ala-Lys-Leu-Leu-Val-Leu-Leu-Thr-Ala-Phe-Val-Ala-Thr-Asp-Ala; (2) Met-Arg-Ser-Leu-Leu-Ile-Leu-Val-Leu-Cys-Phe-Leu-Pro-Leu-Ala-Ala-Leu-Gly; and (3) Met-Arg-Phe-Pro-Ser-Ile-Phe-Thr-Ala-Val-Leu-Phe-Ala-Ala-Ser-Ser-Ala-Leu-Ala.

36. A composition comprising recombinant parathyroid hormone (hPTH) (1-84), wherein the hPTH is made by a process comprising the steps of:

- (a) providing a microorganism comprising:
 - (1) a leader sequence; and
 - (2) a DNA sequence encoding hPTH comprising a functional signal sequence encoded by an amino-terminal amino acid sequence, the expression product of which can direct secretion in yeast, wherein the leader sequence and the hPTH sequence are operably linked;
- (b) culturing said microorganism to allow expression of said DNA sequence encoding hPTH, thereby producing hPTH (1-84); and

(c) purifying the resultant hPTH (1-84) protein.

37. The composition of claim 36, wherein the signal sequence is encoded by the following amino acid sequence:

Met-Asn-Ile-Phe-Tyr-Ile-Phe-Leu-Phe-Leu-Ser-Phe-Val-Gln-Gly-Thr-Arg-Gly.--

REMARKS

Applicants respectfully request formal examination of this application.

I. STATUS OF THE CLAIMS

Claims 1-20 have been cancelled, without disclaimer or prejudice thereof, and claims 21-37 have been added to the application. Applicants reserve the right to prosecute the subject matter of the cancelled claims in this or another application.

New claims 21-23 are directed to a composition comprising human parathyroid hormone (hPTH) (1-84), wherein the hormone has a point mutation at amino acid 26 changing the amino acid from lysine (K26) to glutamine (Q26). The resultant hPTH is superior to wild-type hPTH in that it is resistant to degradation. *See e.g.*, page 17, lines 7-15, of the Application.

Claims 24-29 are directed to a composition comprising hPTH (1-84), wherein the hormone is made using a microorganism comprising the sequence for hPTH and, as a leader sequence, a modified sequence for *Saccharomyces* mating factor $\alpha 1$ (MF $\alpha 1$). For claims 24-26, the MF $\alpha 1$ sequence lacks the yeast STE13 recognition site, and for claims 27-29, the MF $\alpha 1$ sequence comprises only the first 19 amino acids of the sequence.

Claims 30-33 are directed to a composition comprising hPTH (1-84), wherein the hormone is made using a microorganism having a leader sequence and a modified hPTH sequence.

Claims 34-37 are directed to a composition comprising hPTH (1-84), wherein the hormone is made using a microorganism having a leader sequence and either an optimized consensus signal sequence or a functional signal sequence.

Exemplary support provided in the specification for the amended claim and the new claims is given in the following table.

CLAIMS	EXEMPLARY SUPPORT IN SPECIFICATION
21	Page 3, lines 25-27; page 17, lines 11-18; page 22, lines 3-27.
22	Page 20, lines 35-38.
23	Page 20, lines 36-38; page 21, line 36, through page 22, line 2.
24	Page 3, lines 27-30; page 14, lines 20-27; and page 35, line 6, through page 36, line 23.
25, 28	Page 3, lines 10-13; and original claim 10.
26, 29, 33	Page 7, lines 30-32; and page 5, lines 19-22.
27	Page 14, lines 31-35; and page 36, line 25, through page 37, line 35.
30	Page 19, lines 4-17; page 39, lines 7-24; page 37, line 36, through page 38, line 4.
31	Page 14, line 36, through page 15, line 11.
32	Page 6, lines 22-28; page 14, lines 15-20.
34	Page 23, line 30, through page 24, line 29.
35	Page 24, line 30, through page 25, line 36.
36	Page 24, lines 2-7; page 25, lines 3-6.
37	Page 25, line 37, through page 26, line 8.

Because the foregoing amendments do not introduce new matter, entry thereof by the Examiner is respectfully requested.

II. SUMMARY OF THE INVENTION

The present invention is directed to a compositions comprising recombinant hPTH. hPTH is an important regulator of calcium metabolism in mammals, and is also related to several mammalian diseases, such as milk fever, acute hypocalcemia, and otherwise pathologically altered blood calcium levels. *See* page 2, lines 15-19, of the Application. Through its action on target cells in bone and kidney tubuli, hPTH increases serum calcium and decreases serum phosphate, while opposite effects are found regarding urinary excretion of calcium and phosphate. *See* page 5, lines 27-31, of the Application. hPTH is useful, for example, as a component of a diagnostic kit or as a therapeutic in human and veterinary medicine. *See* page 2, lines 20-22, of the Application.

Prior to the present invention, hPTH was commercially available only in very small quantities at high cost, partly because synthesis of the compound was difficult and complex.

See page 1, lines 33-38, of the Application. Moreover, recombinant production of hPTH was hampered by the discovery that *E. coli* degrades human hPTH.

Applicants have overcome the problems of the prior art and discovered compositions of recombinant hPTH that can be made in high yield using microorganisms.

The present invention is directed to a composition comprising a modified recombinant hPTH, wherein the modified hPTH is degradation resistant (claims 31, 36, and 37). See page 15, lines 18-28, of the Application. The modified hPTH has a correct size, full immunological reactivity with two different specific hPTH antibodies, a correct N-terminal amino acid sequence, and comparable biological activity to the wild-type hPTH. See page 15, lines 31-38, of the Application.

In addition, the invention is directed to compositions comprising recombinant hPTH, wherein the hPTH is prepared in microbial cells (claims 24-37). The compositions are made by a process in which (1) the microorganism comprises a modified sequence for *Saccharomyces* MF α 1 (claims 24-29); (2) the microorganism comprises a modified hPTH sequence (claims 30-33); and (3) the microorganism comprises either an optimized consensus signal sequence or a functional signal sequence (claims 34-37). See e.g., page 14, lines 20-27 and 31-35; page 19, lines 4-17; page 23, line 30, through page 24, line 29; page 24, lines 2-7; page 24, line 30, through page 25, line 6; and page 35, line 6, through page 37, line 35, of the specification. Such compositions are superior to those known prior to the claimed invention. Compositions according to the claimed invention can be made in dramatically increased yields of, for example, 5- to 10-fold greater than the prior art. See e.g., page 5, lines 6-12; page 14, lines 23-27; and page 17, lines 7-11, of the specification.


III. CONCLUSION

Applicants respectfully request formal examination of the present application in view of the above amendments and remarks. This application is now in condition for allowance and early notice to that effect is respectfully solicited.

Should the Examiner have any questions or comments regarding the pending application or this Amendment, the Examiner is requested to call the undersigned at 202-672-5538.

If there are any fees due in connection with the filing of this Preliminary Amendment, please charge the fees to our Deposit Account No. 19-0741. If a fee is required for an extension of time under 37 C.F.R. § 1.136 not accounted for above, such an extension is requested and the fee should also be charged to our Deposit Account.

Respectfully submitted,


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Dated: April 7, 1999

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**PRODUCTION OF HUMAN PARATHYROID HORMONE FROM
MICROORGANISMS**

5 This is a Divisional application of prior
application Serial No. 08/087,471, filed on July 2,
1993, which is a File Wrapper Continuation of Serial No.
07/821,478, filed on January 15, 1992, which is a
Continuation of Serial No. 07/404,970, filed on
10 September 8, 1989, now abandoned, which is a
Continuation-In-Part of Serial No. 07/393,851, filed on
August 14, 1989, which issued as U.S. Patent No.
5,010,010 on April 23, 1991, which application, in turn,
is a File Wrapper Continuation of Serial No. 06/921,684,
filed on October 22, 1986, abandoned.

15 **FIELD OF THE INVENTION**

This invention relates to genetically
engineered microorganisms containing DNA coding for
human preproparathyroid hormone.

BACKGROUND OF THE INVENTION

20 This application is a continuation-in-part of
Application Serial No. 07/393,851 filed August 14, 1989,
which is a continuation of Application Serial
No. 06/921,684 filed October 22, 1986, now abandoned.

A number of proteins and peptides that are
25 normally synthesized by mammalian cells have proven to
have medical, agricultural and industrial utility.
These proteins and peptides may be of different
molecular size and have a number of different functions,
for example, they may be enzymes, structural proteins,
30 growth factors and hormones. In essence both proteins
and peptides are composed of linear sequences of amino
acids which form secondary and tertiary structures that
are necessary to convey the biological activity. Human
parathyroid hormone has a relatively small molecular
35 weight, which has made it possible to synthesize the
peptide chemically by the sequential addition of amino
acids. Thus, parathyroid hormone is commercially
available, but in very small quantities at high cost.

As a result, there is no human parathyroid hormone available at a reasonable price to supply the many potential medical, agricultural and industrial applications.

5 During the past ten years, microbiological techniques employing recombinant DNA have made it possible to use microorganisms for the production of species-different peptides. The microorganism is capable of rapid and abundant growth and can be made to
10 synthesize the foreign product in the same manner as bacterial peptides. The utility and potential of this molecular biological approach has already been proven by microbiological production of a number of human proteins that are now available for medical and other uses.

15 Parathyroid hormone (PTH) is one of the most important regulators of calcium metabolism in mammals and is also related to several diseases in humans, animals, e.g. milk fever, acute hypocalcemia and otherwise pathologically altered blood calcium levels.
20 This hormone therefore will be important as a part of diagnostic kits and will also have potential as a therapeutic in human and veterinary medicine.

 The first synthesis of DNA for human preproparathyroid hormone was described by Hendy, G.N.,
25 Kronenberg, H.M., Potts, Jr. J.T. and Rich, A. 78 Proc. Natl. Acad. Sci. 7365-7369 (1981). DNA complementary in sequence to PTH mRNA was synthesized and made double stranded (Hendy et al. supra). This cDNA was cloned in pBR 322 DNA and E. coli 1776 was transfected. Of the
30 colonies with correct antibiotic resistance, 23 out of 200 clones were identified as containing specific human PTH cDNA inserts. However, none of the 23 human PTH clones contained the full length insert (Hendy et al., supra). Later Breyel, E., Morelle, G., Auf'mkolk, B.,
35 Frank, R., Blocker, H. and Mayer, H., Third European Congress on Biotechnology, 10-14 September 1984, Vol. 3, 363-369 described the presence of the human PTH gene in a fetal liver genomic DNA library constructed in the

phage Charon 4A. A restriction enzyme fragment of the PTH gene was recloned and transfected into E. coli.

However, the work of Breyel, supra, demonstrated that E. coli degrades human PTH. Thus, a microorganism which shows a stable production of intact human parathyroid hormone has so far not been described. Further, parathyroid hormone has never before been isolated from yeast.

SUMMARY OF THE INVENTION

Accordingly, it is an object of the present invention to provide a plasmid containing DNA coding for human preproparathyroid hormone (hPTH) for insertion in Escherichia coli. It is another object of the present invention to provide a genetically engineered E. coli containing DNA coding for human preproparathyroid hormone.

A further object of the present invention is to provide a plasmid for insertion in yeast containing DNA coding for parathyroid hormone ("PTH"). It is also an object of the present invention to provide a transformed yeast containing DNA coding for parathyroid hormone including human parathyroid hormone, and from which transformed yeast, parathyroid hormone may be obtained.

Another object of the present invention is to provide new polymers having parathyroid hormone activity including PTH fragments, extension and analogs. Yet another object is to provide alternate leader sequences and secretion signal sequences which can be used in the practice of the present invention.

A still further object of the invention is to provide downstream process technology for purification of intact PTH, as well as purification of analogs, fragments and extensions.

Other objects and advantages of the present invention will become apparent as the description thereof proceeds.

In satisfaction of the foregoing objects and advantages, there is provided by the present invention a novel plasmid for insertion in *E. coli*, containing DNA coding for human preproparathyroid hormone. The plasmid when inserted into *E. coli* functions to transform the *E. coli* such that the *E. coli* then produces multiple copies of the plasmid and thus of the cDNA coding for human preproparathyroid hormone. The plasmid for human insertion into *E. coli* of the present invention and thus the transformed *E. coli* are distinguishable over prior art plasmids and microorganisms, for example as described in Hendy et al., supra, in that the plasmid of the present invention contains a double start codon at the 5' end of the DNA coding for preproparathyroid hormone. The presence of the double start codon may cause a production microorganisms transformed with a plasmid containing the cDNA to produce preproparathyroid hormone at an increased rate and in an improved yield over prior art transformed microorganisms.

There is further provided by the present invention a plasmid for insertion into yeast containing DNA coding for parathyroid hormone. In a preferred embodiment, this plasmid is prepared by recloning the plasmid for insertion in *E. coli* described above. Moreover, the invention provides a yeast transformed by said plasmid for insertion in yeast such that the yeast produces and secretes parathyroid hormone. Thus, the invention provides a method by which parathyroid hormone may be isolated from yeast culture medium. In a preferred embodiment, the transformed yeast is *Saccharomyces cerevisiae*. In another preferred embodiment, the parathyroid hormone is human parathyroid hormone.

By use of in vitro mutagenesis, the present invention also provides substitution of one or more amino acids in human parathyroid hormone and peptides having parathyroid hormone agonistic or antagonistic activity. Further, there are provided analogs,

fragments, or extensions of the parathyroid hormone (collectively referred to as "derivatives") which also show agonistic or antagonistic activity. Examples of these peptides have been produced as secretory products in yeast and in *E. coli*.

The present invention further provides different leader sequences and secretion signal sequences that may be used for the production and secretion of the PTH hormone and/or its derivatives. In at least one instance, an alternate leader sequence provides improved production of the desired hormone or derivative.

Additionally, the invention provides a downstream process technology for purification of human parathyroid hormone and derivatives. The process involves a purification procedure yeast or *E. coli* medium or periplasmic solution, and consists principally of cation exchange chromatography followed by two steps of high pressure liquid chromatography. The final product is more than 95 percent pure and can be submitted directly to N-terminal amino acid sequencing as well as amino acid composition determination.

Human parathyroid hormone (hPTH) is a key regulator of calcium homeostasis. The hormone is produced as a 115 amino-acid prepro-peptide. Before secretion the prepro part is cleaved off, yielding the 84 amino acid mature hormone. Through its action on target cells in bone and kidney tubuli, hPTH increases serum calcium and decreases serum phosphate, while opposite effects are found regarding urinary excretion of calcium and phosphate. At chronically high secretory rates of PTH (hyperparathyroidism) bone resorption supersedes formation. However, prolonged exposure to low/moderate doses of a biologically active PTH-fragment stimulates bone formation and has also been reported to be effective in the treatment of osteoporosis by inducing an anabolic response in bone (Reeve et al. 1980 *Br Med J* 250, 1340-1344; Slovik et al. 1986 *J Bone Min*

Ros 1, 577). So far studies on intact hPTH have been hampered by the limited availability and the high price of the hormone. Hence a system for the efficient expression of hPTH in microorganisms would be very advantageous for the further progression of studies on hPTH and its role in bone biology and disease.

Poly (A)⁺-selected RNA was isolated from human parathyroid adenomas immediately after surgery. The RNA was size-fractionated, cDNA was prepared and cloned into the PstI site of pBR322 by the GC-tailing method. The library was screened by using synthetic oligonucleotides. Sixty-six clones of a total of 34,000 were found to be positive for both 5' and 3' PTH sequences. The correct identity of four of these clones was verified by DNA sequence analysis.

Employing the promoter and signal sequence of *Staphylococcus aureus* protein A we have expressed hPTH in *Escherichia coli* as a secretory peptide. Immunoreactive PTH was isolated both from growth medium and periplasmic space. We obtained up to 10 mg/l hPTH as judged by reactivity in radioimmunoassay.

hPTH was expressed in *Saccharomyces cerevisiae* after fusing hPTH cDNA to an expression vector coding for the prepro-region of the yeast mating factor α . During the secretion process, the α -factor leader sequence is cleaved off by an endopeptidase specific for a dibasic amino acid sequence and encoded by the KEX2 gene.

By hPTH-specific radioimmunoassay a significant amount of hPTH immunoreactive material was detected in the growth medium, corresponding to about 1 mg hPTH per 1 medium, of the yeast strain FL200 transformed with fusion plasmid p α LXPTH. No immunoreactive hPTH was secreted from cells transformed with the vector p α LX.

Parallel cultures of the yeast strain FL200 transformed with one of the three expression plasmids: pUCXPTH, p α UXPTH-1 and p α LXPTH with copy numbers near

unity, normal high (~30) and very high (>50) respectively were grown and both growth medium, a periplasmic fraction and an intracellular soluble fraction were assayed for hPTH immunoreactive peptides.

5 The results show that the intermediate copy number gave the highest production. The produced PTH was secreted completely to the growth medium. The secreted products were concentrated from the growth medium and analyzed on SDS-PAGE. A distinct band with
10 the same molecular weight as hPTH standard was visible on the gel.

 hPTH immunoreactive material was concentrated from the growth medium by passage through a S Sepharose Fast flow column and eluted quantitatively. Recombinant
15 hPTH was purified by reverse phase HPLC. The column was eluted with a linear gradient of acetonitrile/trifluoroacetic acid. A major peak (fractions 32 and 33) with the same retention time as standard hPTH(1-84) was resolved into two peaks in a
20 second HPLC purification step. The major peak from the 2.HPLC eluted exactly as standard hPTH(1-84) and co-chromatographed with hPTH(1-84) as one symmetric peak. SDS-PAGE of the peak fraction showed one band co-migrating with hPTH standard suggesting that the
25 recombinant PTH was essentially pure. The recombinant hPTH was subjected to N-terminal amino acid analysis. We were able to determine unambiguously 45 amino acids from the N-terminal end in the E. coli protein and 19 amino acids in the yeast protein. The sequence was
30 identical to the known sequence of hPTH. The sequence analysis indicated that the recombinant PTH was more than 90 percent pure. The recombinant hPTH from E. coli and *Saccharomyces cerevisiae* was fully active in adenylate cyclase assay and also induced hypercalcemia
35 in rats after injection.

 We have successfully expressed biologically active intact human parathyroid hormone as a secretory peptide in *Escherichia coli* and *Saccharomyces*

cerevisiae, and developed a down-stream purification technology.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 shows all possible variations of the DNA sequence coding for human preproparathyroid hormone.

Figure 2 shows the specific human preproparathyroid hormone DNA coding sequence of the clone pSShPTH-10.

10 Figure 3 shows a DNA sequence coding for human preproparathyroid hormone and having a double start codon at the 5' terminal end with flanking sequences in which are shown all possible variations of the DNA which may be present on the plasmid of the present invention.

15 Figure 4 shows the specific human preproparathyroid hormone DNA coding sequence of the clone pSSHPTH-10 with flanking sequences.

Figure 5 shows the actual amino acids sequence of the human preproparathyroid hormone for which the DNA sequence in close pSShPTH-10 codes.

20 Figure 6 shows the sequence of the MF α 1-hPTH fusion gene with all possible combinations of the DNA coding for hPTH.

Figure 7 shows the sequence of the MF α 1-hPTH fusion gene.

25 Figure 8. Analysis of expression products by SDS-PAGE and immonoblotting.

Saccharomyces cerevisiae transformed with a PTH cDNA carrying plasmid was grown in liquid culture medium. The secreted products were concentrated and analyzed on SDS-PAGE. Panel a shown a silver stained gel with molecular size marker (lane S), hPTH standard (lane P), and concentrated yeast growth medium (lane 1). After blotting onto a PVDF membrane, blots were probed with hPTH specific antibodies, one reactive against the aminoterminal part of the hormone (panel b), another reactive against the middle region of the hormone (panel c). Lanes in panel b and c are numbered as in panel a.

30

35

Figure 9. Purification of recombinant hPTH from the growth medium.

- A: Chromatogram of the 1.HPLC purification
- 5 B: Chromatogram of the 2.HPLC purification of fractions 32 and 33 from panel A. The peak of the recombinant hPTH is indicated by black.
- C: 2.HPLC run of 1 ug standard hPTH(1-84)
- D: Co-chromatography of the recombinant PTH pack from panel B and 1 ug of standard hPTH (1-84)
- 10 E: Silver staining of SDS-PAGE of the proteins in the hPTH pack
- 1: recombinant hPTH, 1 ug
- 2: hPTH(1-84) (σ), 3ug (Note HMW Impurities)

Figure 10. Construction of PPTH-M13- Δ EA/KQ.

- 15 Figure 11. Schematic representation of the mutation introduced in the gene fusion between the yeast α -factor prepro region and the human parathyroid hormone.

- Figure 12. SDS PAGE of concentrated yeast growth medium containing mutated and wild type hPTH.
- 20 Aliquots of concentrated growth medium from yeast strain BJ1991 transformed with the expression plasmids p α UXPTH-2⁹ (lane 2) and p α UXPTH-Q26 (lane 1) were analyzed by 15% PAGE in the presence of 0.1% SDS, and visualized by
- 25 silver staining as described in Experimental Protocol. Lane M shows a molecular size marker including a hPTH standard. The latter is marked with an arrow.

- Figure 13. Purity of purified hPTH (1-84,Q26). Yeast growth medium from yeast strain BJ1991
- 30 transformed with the expression plasmids p α UXPTH-Q26 were concentrated and purified by reversed phase HPLC as described in Experimental Protocol. The purity of the recombinant hormone was then analyzed by analytical HPLC (Panel A) and SDS PAGE (Panel B, lane 2). In Panel B
- 35 the purified hPTH (1-84,Q36) is compared with the wild type hormone purified by two runs on HPLC (lane 3). The molecular weight market in lane M is the same as in

Figure 2. Lane 1 shows a reference PTH produced in *E. coli*.

Figure 14. Two dimensional gelelectrophoretic analysis of hPTH (1-84,Q26). An aliquot of concentrated growth medium from yeast strain BJ1991 transformed with the expression plasmids p α UXPTH-Q26 was separated on an acetic acid 15% PAGE. The two main bands (band 1 and 2) migrating close to the hPTH standard were then cut out, equilibrated with SDS loading buffer and run into a second dimension 15% PAGE containing 0.1% SDS in separate lanes in triplicate. This gel was divided in three and one part was colored with silver (Panel A), one part blotted and treated with hPTH N-terminal region specific antibodies (Panel B) and one part blotted and treated with hPTH middle-region specific antibodies (Panel C). Lanes 1 and 2 show band 1 and 2, PTH_e is a reference hPTH produced in *E. coli*, PTH_c is a commercial hPTH reference. Lane S shows a molecular weight standard.

Figure 15. Biological activity of hPTH (1-84,Q26). Recombinant hPTH (1-84,Q26) (■) was purified on HPLC and assayed for biological activity in a hormone-sensitive osteoblast adenylate cyclase (AC) assay as described in Materials and Methods. The experiments were carried out in triplicate determinations. hPTH (1-84) from Sigma (O) and recombinant yeast hPTH (1-84) (▲) were used as references.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As indicated above, the present invention is directed to a plasmid for insertion in *E. coli* containing DNA coding for human preproparathyroid hormone. The invention is also directed to the resulting transformed *E. coli*.

The invention further is directed to a plasmid for insertion into yeast which contains DNA coding for parathyroid hormone and which is derived from the plasmid for insertion into *E. coli*. Finally, the

invention is directed to a transformed yeast from which parathyroid hormone may be recovered.

The invention further provides methods of producing and isolating the plasmids and transformed microorganisms. Poly(A) selected RNA was isolated from human parathyroid adenomas collected immediately after surgery. The poly(A) RNA was enriched for correct size mRNA by ultracentrifugation through sucrose gradients. Preproparathyroid hormone of correct molecular weight was translated in vitro from this size fractionated poly(A) RNA as judged by sodium dodecylsulphate polyacrylamide gel electrophoreses after immuno precipitation with antiparathyroid antiserum. The specific messenger RNA for the human PTH was used as template for complementary DNA synthesis using oligo d(T)18 as a primer and avian myoblastosis virus reverse transcriptase. After removal of the RNA templates by alkali hydrolysis, the second strand complementary DNA was synthesized by incubating the purified first strand DNA in the presence of the Klenow fragment of E. coli DNA polymerase I. The double stranded complementary DNA was made blunt ended by the action of Aspergillus oryzae single strand specific endonuclease S1 and complementary DNA longer than 500 base pairs was isolated after neutral sucrose gradient centrifugation. Approximately 20 bases long d(C)-tail protrusions were enzymatically added to the 3 ends of the cDNA. This modified complementary DNA was annealed to restriction endonuclease PstI cleaved and d(G)-tailed vector pBR322. Resulting recombinant plasmid DNA's were transformed into E. coli KI2 BJ 5183. Positive transformants were analyzed for by colony hybridization using two different synthetic deoxyribooligonucleotides which covered the N-terminal coding region as well as the 3' non-coding part of the hormone mRNA sequence, respectively. Six out of 66 clones that were positive for both probes were submitted for detailed analysis by restriction endonuclease mapping showing that they all were

identical except for some size heterogeneity at the regions flanking the start codon and the XbaI site 3' for the stop codon. One clone, pSShPTH-10, was subjected to DNA sequence analysis revealing a
5 432 nucleotide long human parathyroid hormone complementary DNA sequence inserted in the PstI site of pBR 322. The entire cDNA sequence was found to be identical to the sequence previously described by Hendy, et al., supra, except for a 5 base pair deletion in
10 front of the start codon.

Figure 2 shows the human preproparathyroid hormone DNA sequence of pSShPTH-10. This may be compared with Figure 1, which shows all possible variations of the DNA sequence for human preproparathyroid
15 hormone without the 5' double start codon. Figure 3 shows the DNA sequence of the clone of the present invention with the flanking sequences. In a preferred embodiment, the plasmid for insertion in E. coli coding for human preproparathyroid hormone is pSShPTH-10, the
20 DNA sequence of which, including the flanking sequence, is shown in Figure 4.

The invention further provides a plasmid for insertion into yeast containing DNA coding for parathyroid hormone. The parathyroid hormone may be
25 human or animal parathyroid hormone, for example pig or bovine parathyroid hormone. The plasmid for insertion in yeast of the present invention may be recloned from plasmids containing DNA coding for human or animal parathyroid hormone. In a preferred embodiment, the
30 plasmid for insertion in yeast contains DNA coding for human parathyroid hormone. As shown in the following examples, the hTPH sequence from pSShPTH-10 has been recloned and inserted in designed vectors for expression in *Saccharomyces cerevisiae*.

35 pSShPTH-10 was digested to form a 288 bp BglIII-XbaI fragment. This fragment was then subcloned into pUC19 between the BamHI and XbaI sites. The subclone was then digested with Dpn I, and the largest

resulting fragment was isolated. The said fragment was then digested with SalI.

The plasmid pSS α LX5-hPTH1 that in yeast MAT cells leads to the expression and secretion of PTH was constructed in three stages:

1. Construction of the yeast shuttle vector pL4 (which replicates in both *E. coli* and *Saccharomyces cerevisiae*).
2. Cloning of a DNA fragment containing the yeast mating pheromone MF α 1 gene and its insertion into the yeast shuttle vector to make the p α LX5 vector.
3. Insertion of a DNA fragment from the coding region of the hPTH gene of pSShPTH-10 into p α LX5 in reading frame with the prepro part of the MF 1 gene, thereby producing the vector pSS α LX5-hPTH1.

The shuttle vector pL4 was constructed by inserting into pJDB207, an EcoRI-AvaII fragment containing the ADHI promoter isolated from PADH040. A SphI fragment was then deleted, resulting in a plasmid pALX1. The PstI site in the B-lactamase gene was deleted and the plasmid was partially digested with PvuI and BglI and ligated to a PvuI BglI fragment of pUC8, to form pALX2. After a further oligonucleotide insertion, the plasmid was digested with HindIII and religated to form pALX4.

Total yeast DNA from the Y288C strain was digested with EcoRI, and the 1.6-1.8 kb fragments isolated. These were ligated to EcoRI-cleaved pBR322, and *E. coli* was transformed. The clones were screened for MF α 1 inserts by oligonucleotide hybridization. The DNA selected thereby was then used to transform *E. coli*. The resulting plasmid pMF α 1-1 was digested with EcoRI, made blunt ended by Klenow enzyme, and then digested with BglIII. The MF α 1 fragment was isolated, and ligated to pL5 (digested with BamHI, made blunt ended with Klenow enzyme, and digested with BglIII) to yield p α LX5.

In order to insert the human PTH cDNA fragment into p α LX5, the p α LX5 was digested with HindIII, creating sticky ends and the site was made blunt ended with the DNA polymerase I Klenow fragment and dNTP. The
5 p α LX5 was then digested with SalI to create a sticky ended DNA complementary to the SalI digested human PTH fragment described above.

The SalI digested human PTH fragment was then inserted into the SalI digested p α LX5. The resulting
10 plasmid pSS α LX5-PTH was then inserted into yeast, thereby transforming yeast so that the yeast produces and secretes intact human parathyroid hormone. In a preferred embodiment, the transformed yeast is *Saccharomyces cerevisiae*.

15 As explained above, the invention provides alternate leader sequences which may be used for the production of parathyroid hormone or derivatives thereof, as taught by the present invention. The method set forth above discloses the use of the α -factor leader
20 sequence. However, other sequences may be used, at least one of which has been shown to process PTH with greater efficiency than does the entire α -factor leader sequence. It has been discovered that the deletion from the α -factor leader of a 12-base sequence which
25 comprises the yeast STE13 recognition site produces a more efficient production mechanism for PTH and/or its derivatives. pSS α UXPTH- Δ EA contains the α -factor hPTH fusion gene placed between the α -factor promoter and terminator, in which the region encoding the Glu-Ala-Glu-Ala
30 recognition sequence of the yeast STE13 aminopeptidase has been deleted. As another example of an alternative leader sequence, a leader sequence comprised of only the first nineteen amino acids of the α -factor is also used in the method of the present
35 invention.

Also shown is an example of site specific mutagenesis changing the codon for the amino acid 26 in the PTH gene, thereby transforming a lysine-codon (K) to

glutamine-codon (Q) using the Muta-Gene[™] in vitro mutagenesis kit from Bio-Rad. For this purpose, the plasmid p α PTH-M13- Δ EA was used to transform the E. coli strain CJ236. A uracil-containing single-stranded DNA
5 which was prepared from the phage was annealed to a synthetic oligonucleotide, and second strain synthesis was carried out with T4 DNA polymerase and ligation with T4 DNA ligase. The heteroduplex DNA was transformed into the E. coli strain MV1190 to be repaired into a
10 homoduplex by removal of uracil incorporated in the parental strand. Positive clones were verified by DNA sequencing and one of these was called p α PTH-M13- Δ EA/KQ. Finally, the entire expression cassette between a BamHI and a filled-in EcoRI site was isolated from this vector
15 construction and inserted into the BamHI and PvuII site of the yeast shuttle vector YEp24 and this final expression plasmid was designated pSS α UXPTH- Δ EA/KQ.

A point mutation was introduced in the gene encoding the human parathyroid hormone leading to a
20 change of the 26th amino acid from Lysine (K26) to Glutamine (Q26). When this gene was expressed and secreted in *Saccharomyces cerevisiae* using the α -factor fusion system, the full length hormone was found in the growth medium with no degradation products present.
25 This contrasts the situation when the wild type gene is expressed in the same system. Then the major product is a hormone fragment hPTH(27-84), and only up to 20% of the immunoreactive secreted material is hPTH(1-84). The yield after a two step purification of the degradation
30 resistant hormone was 5-10 fold higher than what was obtained with the wild type hormone. The secreted hPTH(1-84,Q26) had correct size, full immunological reactivity with two different hPTH specific antibodies and correct N-terminal amino acid sequence.
35 Furthermore, the introduced mutation had no effect on the biological activity of the hormone as judged from its action in a hormone-sensitive osteoblast adenylate cyclase assay.

Human parathyroid hormone (hPTH) is one of the key calcium regulating hormones in the body. The hormone is produced in the parathyroid gland as a 115 amino acid prepro-peptide that is processed during secretion to an 84 amino acid mature hormone.^{1/} It acts primarily on kidney and bone cells, stimulating calcium back resorption and calcium mobilization, respectively.^{2-4/} The hormone seems to exhibit differential catabolic as well as anabolic effects and its overall physiological action is probably to generate a positive calcium balance and enhance bone formation. The area of potential utility includes possible use in treatment of postmenopausal osteoporosis as well as in prevention of postpartum hypocalcaemia in cows. Sufficient supplies of authentic recombinant hPTH are of considerable interest to evaluate such applications.

hPTH is an easily degraded polypeptide. Already in the parathyroid gland large amounts of carboxyl-terminal PTH fragments are generated.^{1/} Structural studies have suggested that hPTH may contain two domains with the easily cleaved region placed in a connecting stalk between these domains.^{5/} Not surprisingly therefore, degradation of hPTH has been a major problem when the hormone is expressed in heterologous organisms. In *E. coli* low expression levels combined with degraded hormone peptides of short half-life were observed.^{6-8/} The most successful expression system for hPTH so far is *Saccharomyces cerevisiae* where the hormone is expressed as a secretory peptide.^{9/} By that approach we were able to obtain significant amounts of authentic hPTH(1-84) with full biological activity. But even if conditions were found which eliminated proteolytic attacks at some sites in the putative stalk region of the hormone, a significant fraction of the secreted peptides was still cleaved after a pair of basic amino acids found in the hPTH sequence reducing the yield of full length peptide hormone. The cleavage site resembles that recognized by

the yscF protease (the KEX2 gene product).^{10,11/} We reasoned that the elimination of the putative yscF cleavage in hPTH could lead to a significant gain in the yield of undegraded hPTH secreted from yeast. In the present report we describe the removal of the putative yscF cleavage sites by *in vitro* mutagenesis of the hPTH coding region. When the amino acid at position 26 in hPTH was changed from Lysine (K26) to Glutamine (Q26), the major degradation product hPTH(27-84) previously observed disappeared in the growth medium and the yield of full-length hormone increased 5- to 10-fold. The secreted degradation resistant hPTH(1-84, Q26) had correct size, full immunological reactivity with two different hPTH specific antibodies and correct N-terminal amino acid sequence. Furthermore, the introduced mutation had no effect on the biological activity of the hormone as judged from its action in a hormone-sensitive osteoblast adenylate cyclase assay.

The *Saccharomyces cerevisiae* strain used for the hPTH expression was BJ1991 (a, *trp1*, *ura3-52*, *leu2*, *prb1-1122*, *pep4-3*). Yeast cells were transformed by the lithium method^{12/}, and transformants grown at 30°C in YNBGC medium (0.67 percent yeast nitrogen base, 2 percent glucose, 1 percent casamino acids (Difco).

The pAUX⁺PTH-2 plasmid used as a reference for expression of authentic hPTH(1-84) is described.^{9/} In order to change the codon 26 in the hPTH gene from AAG (Lysine) to CAG (Glutamine), an α -factor hPTH gene fusion subcloned in M13 mp19 (designated M13PTH-3 in ^{9/}) was modified by *in vitro* mutagenesis using the "Muta-gene" *in vitro* mutagenesis kit" (Bio-Rad) based on the method of Kunkel et al.^{13/}. The mutagenizing oligonucleotide had the sequence 5'-GGCTGCGTCAGAAGCTGC-3' where all nucleotides except the ninth are complementary to the actual hPTH sequence. Positive clones were verified by DNA sequencing.^{14/} One of those were picked and called M13PTH-Q26. The entire expression cassette between a BamHI and a filled in

EcoRI site was finally isolated from M13PTH-Q26 and inserted between the BamHI and PvuII site of the yeast shuttle vector YEp24.^{15/} This expression plasmid was designated paUXPTH-Q26. The translation product from the hPTH gene between amino acid 25 and 27 should now change from Arg-Lys-Lys to Arg-Gln-Lys.

Radioimmunoassay of hPTH in yeast culture media was carried out as described.^{9/,16/}. For electrophoretic analysis, yeast culture media were concentrated as previously described^{9/}, and separated on a 15 percent polyacrylamide gel in the presence of SDS^{17/}, and either stained with silver^{18/} or further analyzed by protein blotting using Immobilon PVDF Transfer Membranes (Millipore) and the buffers of Towbin et al.^{19/} Reference hPTH(1-84) was purchased from Peninsula Laboratories (USA). Protein blots were visualized as described.^{9/}

The concentrated medium from the Sepharose S column was subjected to further purification by reversed phase HPLC using a Vydac protein peptide C18 column (The Separation Group, Hesperia, CA, USA). The column was eluted with a linear gradient of acetonitrile/0.1 percent trifluoroacetic acid.

Proteins to be sequenced was purified either by HPLC as described above or by SDS polyacrylamide gelelectrophoresis followed by blotting onto polyvinylidene difluoride membranes.^{20/} Automated Edman degradation was performed on a 477A Protein Sequencer with an on-line 120A phenylthiohydantoin amino acid analyzer from Applied Biosystems (Foster City, CA, USA). All reagents were obtained from Applied Biosystems.

The adenylate cyclase stimulating activity of the recombinant hPTH was assayed as previously described^{9,21,22/} hPTH(1-84) from Sigma was used as reference.

Different strategies could be envisaged to avoid the degradation of parathyroid hormone during expression in heterologous organisms. One recently

reported strategy is to express intracellularly in E. coli a cro-lacZ-hPTH fusion protein that subsequently is cleaved by strong acid to give proline-substituted hPTH.^{23/} However, since secretion of the hormone in yeast seems to be a more efficient way of producing a correctly processed hormone, and also is superior with respect to downstream processing, we rather adopted a strategy to improve this system. Only one major cleavage site is used during secretion in yeast when the cells are grown under proper conditions: after a pair of basic amino acids in position 25 and 26 in the hPTH sequence. This cleavage site resembles that recognized by the yscF protease (the KEX2 gene product). We reasoned that a substitution of a glutamine for the lysine 26, as illustrated in Fig. 11, ought to be a structurally conservative change that should exclude the hormone as a substrate for the yscF protease.

The yeast strain BJ1991 was transformed with the plasmids paUXPTH-Q26 containing the mutated hPTH coding region. One transformant was grown in YNBGC medium lacking uracil and the cell free medium was concentrated and analyzed in different gel systems. Figure 12 shows a silver-stained SDS polyacrylamide gel where concentrated medium from paUXPTH-Q26 transformed cells (mutated hPTH, lane 1) is compared with that from paUXPTH-2 transformed cells (wild type hPTH, lane 2). In the latter case the strongest band has a molecular mass lower than the standard hPTH, and previous microsequencing has shown that it corresponds to the hormone fragment hPTH(27-84). In the lane with the mutated product (lane 1), this band is absent showing that the cleavage between amino acid 26 and 27 has been totally eliminated as a result of the mutation. Now the major product is a polypeptide that migrates close to the full length hPTH standard. Consistently, this band had a migration slightly faster than the standard in an anionic gel system and a migration slightly slower than the standard in a cationic gel system in accordance with

the single charge difference between the mutated (one positive charge less) and the wild type hormone. In addition to the main product a few weaker bands were present of apparently higher molecular mass which might
5 be O-glycosylated forms of the hormone.

This hPTH(1-84,Q26) candidate was further analyzed by two dimensional gel electrophoresis and protein blotting. In the first dimension acetic acid/urea gel a simple pattern with mainly two bands was
10 found. These were cut out and run on a second dimension SDS polyacrylamide gel. The silver stained second dimension gel as well as two protein blots probed with different PTH antibodies, are shown in Figure 14. The hPTH(1-84,Q26) candidate migrating closest to the hPTH
15 standard in both dimensions, reacted with two hPTH specific antibodies raised against N-terminal region and the middle/C-terminal region of the hPTH respectively.

The nature of the hPTH(1-84,Q26) candidate was finally confirmed by N-terminal amino acid sequencing, both directly on the polypeptide band after blotting
20 onto a PVDF membrane filter, and after purification on reversed phase HPLC. Correct amino-terminal sequence was found in both cases. Furthermore, the expected change from lysine to glutamine in position 26 was
25 confirmed by sequencing through this position.

Since the elimination of the internal cleavage of the secreted hPTH leads to fewer polypeptides with similar properties in the growth medium, this form of the hormone could also be isolated by a simplified
30 purification procedure. Already in the first concentration step using a Sepharose S column, a certain purification is achieved. All hPTH immunoreactive material is retained, but some high molecular weight material is removed in the pH6 wash of the Sepharose S
35 column. This first concentrated eluate already contained more than 80 percent hPTH(1-84, Q26). Then, a single run on a reversed phase HPLC C18 column, was enough to give near homogeneous hPTH(1-84, Q26). The

5 purity was checked both by SDS polyacrylamide
gelelectrophoresis and sensitive silver-staining, and by
analytical HPLC as illustrated in Figure 13A. A single
peak is found in the chromatogram (Figure 13A), and a
single band with only a trace of a closely migrating
hPTH band (probably an O-glycosylated form of the
hormone) could be seen in the SDS polyacrylamide gel
(Figure 13B). When the yield of pure full length
mutated hormone was compared with that of the wild type,
10 5 to 10 fold higher yields were usually achieved. This
is consistent with our previous estimate of the fraction
of full length hormone (up to 20 percent) obtained when
the wild type is expressed.^{9/}

15 The biological activity of the secreted
hPTH(1-84, Q26) was tested in a hormone-sensitive
osteoblast adenylate cyclase assay.^{9,21,22/} The
purified hPTH(1-84, Q26) was analyzed for its ability to
stimulate the adenylate cyclase activity of OMR 106
osteosarcoma cells above the basal level. The
20 quantitative analysis shown in Figure 15, clearly
demonstrates that hPTH(1-84, Q26) has a stimulatory
effect comparable to that of a commercial hPTH control.
The stimulation curve practically coincides with that of
purified recombinant wild type hPTH(1-84).
25 Consequently, no difference in biological activity could
be detected between the wild type hormone and the
degradation resistant mutated hormone.

30 We have shown that the easily degraded human
parathyroid hormone can be expressed in a correctly
processed and intact form in *Saccharomyces cerevisiae*
after the introduction of a single, structurally
conservative mutation in the 26th amino acid of the
hormone. The increase in final yield of pure full
length hormone is 5- to 10-fold compared to what is
35 obtained with wild type hormone expressed in the same
system. The mutation also simplifies the downstream
purification of the hormone. A concentration step

followed by a single HPLC run was enough to give near homogeneous recombinant hormone.

We have previously described conditions of growth that eliminates secondary cleavages in the protease sensitive "stalk" region of the hormone ^{9/}. Here we describe how the final dibasic cleavage site can be eliminated. After introduction of the mutation, a form of the hormone is produced that totally resists the frequent cleavage found in the wild type hormone after the Arg25-Lys26 motif. The possible internal cleavage at putative dibasic amino acids is one of the severe drawbacks of the α -factor secretion system. To our knowledge this is the first reported case where this problem has been successfully overcome.

Previous reports have shown that the biological activity of the hormone resides in the first third of the molecule in a minimum structure comprised of amino acids 1-27. Furthermore, the triple basic amino acid motif from position 25-27 seems to be conserved between the bovine^{25/}, porcine^{26/} and human hormone^{27/}. It was therefore not obvious that the introduction of a mutation in position 26 would not destroy the biological activity of hPTH. However, no difference between the recombinant hPTH products could be detected in the adenylate cyclase assay, showing that the introduced mutation does not affect the biological activity of the hormone.

hPTH is a multifunctional hormone with many potential uses, for example in diagnostics and as a drug in veterinary medicine. A fragment of hPTH together with 1,25(OH)₂ vitamin D₃ has also been reported to induce bone formation in humans ^{27, 28/}, and one of the major areas of potential use of a recombinant hPTH is therefore in the treatment of osteoporosis. To evaluate such applications, sufficient supplies of recombinant hPTH are essential. In the present report we have described what we believe is the most efficient way of

producing full length biologically active parathyroid hormone so far.

Moreover, the method of the present invention may be used to produce parathyroid hormone derivatives having parathyroid hormone agonistic or antagonistic activity. These derivatives include hormone analogs, such as the example described above in which the lysine at position 26 is substituted with glutamine, or may be fragments or extensions of the hormone, i.e., polypeptides having parathyroid hormone agonist or antagonist activity which are respectively shorter or longer than the hormone itself. Parathyroid hormone agonistic effect in this connection will be demonstrated by activation of adenylyl cyclase in bone cells and kidney cells. The in vivo effects of such activity mimic the effects of native parathyroid hormone with respect to plasma calcium concentration alterations as well as the well known hormonal actions on calcium and phosphate re-absorption and excretion in the kidney. Furthermore, the PTH derivatives of the present invention having agonist activity shall also have the capacity to reduce the alkaline phosphatase activity of certain osteoblast cell lines, and stimulate ornithine decarboxylase activity bone cells (UMR 106 cells) or chicken condrocytes and stimulate DNA synthesis in chicken condrocytes. Moreover, the derivatives shall have the capability of blocking the action of parathyroid hormone itself or of any of the other agonist derivatives.

The invention also provides alternate secretion signal sequences for the secretion of the PTH hormone or its derivatives from yeast. As disclosed above, parts of the MF α 1 gene may be inserted into the plasmid of the present invention to cause the yeast to secrete the intact PTH hormone or derivatives. However, other signal sequences will also function in the methods of the present invention. The process of protein secretion requires the protein to bear an amino-terminal

signal peptide for correct intracellular trafficking, the sequence of which is termed "signal sequence". Two classes of signal sequences will function in the plasmids of the present invention, and will cause secretion of the PTH hormone or derivative from yeast: "optimized consensus signal sequences" and other functional signal sequences. An "optimized consensus signal sequence" is any amino-terminal amino acid sequence that is composed of the following three parts:

1. An amino-terminal positively charged region. The size of this region may vary from 1-20 amino acids. The only specific characteristic is a positive charge at physiological pH conferred by the presence of one to three basic amino acids (Lys or Arg).

2. A hydrophobic core region. The size of this region may vary from 7-20 amino acids, and it is predominantly composed of hydrophobic amino acids (Phe, Ile, Leu, Met, Val, Tyr, or Trp).

3. A polar COOH-terminal region composed of five amino acids (from position -5 to -1 relative to the cleavage site) that defines the cleavage site. The specific character of this region is that the amino acid in position -1 must be a small neutral amino acid (Ala, Ser, Gly, Cys, Thr, or Pro), and that the amino acid in position -3 must be either a hydrophobic amino acid (Phe, Ile, Leu, Met, Val) or a small neutral amino acid (Ala, Ser, Gly, Cys, Thr, or Pro).

See von Heijne, G. (1983) "Patterns of Amino Acids near Signal-Sequence Cleavage Sites." Eur. J. Biochem. 133, 17-21, and von Heijne, G. (1985) "Signal sequences. The limits of variation." J. Mol. Biol. 184, 99-105. However, Kaiser, C.A., Preuss, D., Grisafi, P., and Botstein, D. (1987) "Many Random Sequences Functionally Replace the Secretion Signal Sequence of Yeast Invertase." Science 235, 312-217, found the specificity with which signal sequences were recognized in yeast to

be low and that any amino-terminal peptide with a hydrophobicity above some threshold value would function. Therefore, "functional signal sequence" is defined as any amino-terminal amino acid sequence that can direct secretion in yeast even if it does not fit all the criteria of an optimal signal sequence.

Specific examples of signal sequences functional in yeast that conform to the description of an optimal signal sequence are:

1. Met,Lys,Ala,Lys-Leu,Leu,Val,Leu,Leu,Thr,Ala, Phe-Val,Ala,Thr,Asp,Ala (Jabbar, M.A., and Nayak, D.P. (1987) "Signal Processing, Glycosylation, and Secretion of Mutant Hemagglutinins of a Human Influenza Virus by *Saccharomyces cerevisiae*." Molec. Cell. Biol. 7, 1476-1485.) from a human influenza virus hemagglutinin.

2. Met,Arg,Ser-Leu,Leu,Ile,Leu,Val,Leu,Cys,Phe, Leu,Pro-Leu,Ala,Ala,Leu,Gly (Jigami, Y., Muraki, M., Harada, N., and Tanaka, H. (1986) "Expression of synthetic human-lysozyme gene in *Saccharomyces cerevisiae*: use of a synthetic chicken-lysozyme signal sequence for secretion and processing." Gene 43, 273-279.) from chicken lysozyme.

3. Met,Arg,Phe,Pro,Ser-Ile,Phe,Thr,Ala,Val,Leu, Phe,Ala,Ala-Ser,Ser,Ala,Leu,Ala (Ernst, J.F. (1988) "Efficient Secretion and Processing of Heterologous Proteins in *Saccharomyces cerevisiae* is mediated solely by the Pre-Segment of α -factor Precursor." DNA 7, 355-360. Kurjan, J. and Herskowitz, I. (1982) "Structure of a Yeast Pheromone Gene (MFa): "A putative α -factor Precursor contains four Tandem Copies of Mature α -factor". Cell 30, 933-934.) from yeast α -factor precursor.

A specific example of signal sequences functional in yeast that conforms to the description of

a functional signal sequence is Met, Asn, Ile, Phe, Tyr, Ile, Phe, Leu, Phe, Leu, Ser, Phe, Val-Gln, Gly, Thr, Arg, Gly. Baldari, C., Marray, J.A.H., Ghiara, P., Cesareni, G., and Caleotti, C.L. (1987) "A novel leader peptide which allows efficient secretion of a fragment of human interleukin 1B in *Saccharomyces cerevisiae*." EMBO J. 6. 229-234. from *Klyveromyces lacis* killer toxin.

Finally, the invention provides three different steps which taken together, represent an effective and convenient procedure for purification of human recombinant parathyroid hormone (PTH). A cation exchange chromatography using S-Sepharose column as described in the text, washed at pH 6 and eluted at pH 8.5. The immunoreactivity of the intact PTH migrates within the peak.

Figure 9 shows high performance liquid chromatography (HPLC) of hPTH which was eluted with trifluoroacetic acid and a linear gradient of acetonitril of 35-60%. The position of intact hPTH is indicated in the second HPLC step the acetonitril gradient has been changed to 40-45% and intact hPTH elutes as one symmetrical peak.

Although the methods of making the invention disclosed herein are shown in detail, these methods are presented to illustrate the invention, and the invention is not limited thereto. The methods may be applied to a variety of other plasmids containing DNA coding for human or animal PTH to produce the plasmids for insertion in yeast of the present invention.

The plasmids of the present invention and transformed microorganisms were produced as set forth in the following examples.

EXAMPLE 1

Isolation of mRNA and synthesis of complementary DNA (cDNA) of human parathyroid hormone.

Starting material for the invention was parathyroid adenomas obtained from patients by surgery.

The parathyroid tissue was placed on dry ice directly after removal and transported to a laboratory for preparation of RNA. The frozen tissue was homogenized with an ultra Turax homogenizer in the presence of 4 M Guanidinium thiocyanate and the RNA content was recovered by serial ethanol precipitations as described by Chirgwin, J.M., Przybyla, A.E., MacDonald, R.J. and Rutter, W.J., 18 Biochemistry 5294-5299 (1979). The RNA preparation was applied to oligo d(T) cellulose affinity chromatography column in order to enrich for poly(A) containing mRNA. The poly(A) rich RNA was further enriched for parathyroid hormone (PTH) mRNA sized RNA by ultracentrifugation through a 15-30% linear sucrose gradient. The resulting gradient was divided into 25 fractions and every third fraction was assayed for PTH mRNA content by in vitro translation followed by immunoprecipitation with anti PTH antiserum (Gautvik, K.M., Gautvik, V.T. and Halvorsen, J.F., Scand. J. Clin. Lab. Invest. 43, 553-564 (1983)) and SDS-polyacrylamide gel electrophoresis (Laemmli, U.K., 227 Nature 680 (1970)). The RNA from the fractions containing translatable PTH mRNA was recovered by ethanol precipitation. This RNA, enriched for PTH mRNA, was used as a template for cDNA synthesis using oligo d(T)18 as a primer and avian myoblastosis virus reverse transcriptase for catalysis of the reaction (Maniatis, T., Fritsch, E.F. and Sambrook, J., Molecular Cloning pp. 230-243 (1982)). After first strand synthesis, the RNA templates were removed by alkali hydrolysis. The second strand cDNA was synthesized by incubating the purified first strand cDNA in the presence of the Klenow fragment of E. coli DNA polymerase I (Maniatis, supra). This in vitro synthesized double stranded cDNA was made blunt ended by the action of *Aspergillus oryzae* single strand specific endonuclease S1 (Maniatis, supra). The blunt ended double stranded cDNA was size fractionated over a 15-30% neutral sucrose gradient. The size distribution of each fraction was estimated by agarose

gel electrophoresis together with known DNA fragment markers. Fractions containing cDNA larger than approximately 500 base pairs were pooled and the cDNA content was collected by ethanol precipitation.

5

EXAMPLE 2

Cloning of cDNA PTH in plasmid pBR 322 and transformation of E. coli K12 BJ5183.

10 An approximate 20 base long d(C)-tail protrusion was enzymatically added to the 3' ends of the cDNA by the action of terminal deoxynucleotidyl transferase (Maniatis, supra). The d(C)-tailed cDNA was annealed to restriction endonuclease Pst I cleaved and d(G)-tailed vector pBR322 and the resulting recombinant plasmid DNA's were transformed into E. coli K12 BJ 5183
15 cells which were made competent by the method of Hanahan, D., 166 J. Mol. Biol. 166, 557-580 (1983). A total of 33,000 transformants were analyzed for PTH cDNA content by colony hybridization (Hanahan, D. and Meselson, Gene 10, 63 (1980)).

20

Two to three thousand transformants were plated directly on each 82 mm diameter nitrocellulose filter, placed on top of rich medium agar plates containing tetracycline, and incubated at 37 degrees Centigrade until approximately 0.1 mm diameter colonies
25 appeared. Duplicate replicas of each filter was obtained by serial pressing of two new filters against the original filter. The replica filters were placed on top of new tetracycline containing agar plates and incubated at 37 degrees Centigrade until approximately
30 0.5 mm diameter colonies appeared. The master filter with bacterial colonies was kept at 4 degrees Centigrade placed on top of the agar plate and the duplicate replica filters were removed from the agar plates and submitted to the following colony hybridization
35 procedure.

EXAMPLE 3

Characterization of bacterial clones containing recombinant cDNA PTH and of the DNA sequence of clone pSSHPTH-10.

5 The cells in the respective colonies were disrupted in situ with alkali and sodium chloride leaving the DNA content of each bacterial clone exposed. The procedure allows the DNA to bind to the filter after which it was neutralized with Tris-buffer and dried at
10 80 degrees Centigrade. The majority of cell debris was removed by a 65 degree Centigrade wash with the detergent sodium dodecylsulphate (SDS) and sodium chloride leaving the DNA bound to the filters at the position of the former bacterial colonies. The filters
15 were presoaked in 6xSSC (0.9 M NaCl, 0.09M Na-citrate), 1x Denhart's solution (0.1 g/ml Ficoll, 0.1 g/ml polyvinyl pyrrolidone, 0.1 g/ml bovine serum albumin), 100 g/ml herring sperm DNA, 0.5% SDS and 0.05% sodium pyrophosphate for 2 hours at 37 degrees Centigrade
20 (Woods, D.E. 6 Focus Vol. No. 3. (1984)).

 The hybridization was carried out at 42 degrees Centigrade for 18 hours in a hybridization solution (6x SSC, 1x Denhart's solution, 20 g/ml tRNA and 0.05% sodium pyrophosphate) supplemented with 32P-labelled DNA probe. (Woods supra).

 The DNA used as a hybridization probe was one of two different synthetic deoxyribo oligonucleotides of which the sequences were deduced from the published human PTH cDNA sequence of Hendy, supra. The first
30 probe was a 24-mer oligonucleotide originating from the start codon region of the human preproPTH coding sequence having a nucleotide sequence reading TACTATGGACGTTTTCTGTACCGA. The second oligonucleotide was a 24-mer spanning over a cleavage site for the
35 restriction endonuclease XbaI located 31 nucleotides downstream of the termination codon and consisted of the nucleotide sequence CTCAAGACGAGATCTGTACATCC.

Labelling was carried out by transfer of 32 P from 32 P- γ -ATP to the 5' end of the oligonucleotides by the action of polynucleotide kinase (Maxam, A.M. and Gilbert, W., 65 Methods Enzymol., 499 (1980)).

5 The hybridized filters were washed in 6xSSC, 0.05% sodium pyrophosphate at 42 degrees Centigrade prior to autoradiography. Sixty-six clones were found to be positive for both probes as judged from hybridization to both copies of the duplicate replica
10 filters. All those were picked from the original filters with the stored cDNA library and amplified for indefinite storage at -70 degrees Centigrade. Six of these were chosen for plasmid preparation and a more
15 detailed analysis by restriction endonuclease mapping, showing that all were identical except for some size heterogeneity at the regions flanking the start codon and Xba I site, respectively.

EXAMPLE 4

Clone pSSHPTH-10.

20 One clone, pSSHPTH-10, was subjected to DNA sequence analysis according to the method of Maxam and Gilbert, supra. This clone consists of a 432 base pair long PTH cDNA sequence inserted in the Pst I site of pBR322 having 27 G/C base pairs at the 5' end and 17 G/C
25 base pairs at the 3' end. The complete DNA sequence of the cDNA insert of pSSHPTH-10 is shown in Figure 4. It is identical to the sequence of Hendy, et al., supra except for a five base pair deletion right in front of the start codon, changing the published (Hendy, supra)
30 start-stop (ATGTGAAG) signal (deletion is underlined) preceding the used start codon (ATG) to a double start signal (ATGATG).

EXAMPLE 5

Construction of the yeast shuttle vector pL4.

35 Before the hPTH-yeast-expression project was initiated, a family of general yeast expression vectors were developed. One of these, pL4, later was used to make pSS LX5-hPTH1, as described below:

The plasmid pJDB207, constructed by Beggs, J.D., "Multiple-copy yeast plasmid vectors," Von Wettstein, D., Friis, J., Kielland-Brandt, M. and Stenderup, A. (Eds) Molecular Genetics in Yeast (1981), Alfred Benzon Symposium Vol. 16, 383-390, was chosen as the basis for the general expression vectors. It contains an EcoRI fragment of the yeast 2 micron DNA inserted into the pBR322 derivative pAT153. It also contains the yeast LEU2 gene. The copy number of pJDB207 in yeast *cir*⁺ cells is very high relative to that of other plasmids and it is unusually stable after non-selective growth in a *cir*⁺ strain, Parent, S.A., Fenimore, C.M., and Bostian, K.A. "Vector Systems for the Expression, Analysis and Cloning of DNA Sequences in *S. cerevisiae*". 1 Yeast 83-138 (1985); Erhart, E. and Hollenberg, C.P., "The Presence of a Defective LEU2 Gene on 2 Micron DNA Recombinant Plasmids of *Saccharomyces cerevisiae* is Responsible for Curing and High Copy Number," 156 J. Bacteriol. 625-635 (1983). These properties are related to a partial defective promoter in the selective marker gene LEU2 (often named LEU2d, d for defective), Erhart et al., supra, which is not changed in the following constructs.

A 1260 base pair EcoRI-AvaII fragment containing the ADHI promoter was isolated from the plasmid pADH040. After a fill in reaction with the Klenow fragment of DNA polymerase I and all four dNTPs, BamHI linkers were attached and the fragment was cloned into the unique BamHI site of pJDB207. From the plasmid with the promoter in a counterclockwise direction, a 1050 base pair SphI fragment was then deleted (from the SphI site in pJDB207 to the SphI site in the promoter fragment) leaving only a single BamHI site. This plasmid was designated pALX1.

Then the PstI site in the B-lactamase gene of pALX1 was eliminated without inactivating the gene. pALX1 was digested to completion with PstI and nuclease S1 to destroy the PstI site, and then subjected to a

partial digestion with PvuI BglI. At the same time a 250 base pair PVUI BglI fragment was isolated from pUC8, Vierira, J. and Messing, J. 19 Gene 259 (1982), that contains the corresponding part of a B-lactamase without a PstI site. This was ligated to the partially digested pALX1. In all the ampicillin resistant clones isolated the B-lactamase gene had been restored by incorporating the pUC8 fragment. This plasmid was called pALX2.

The following oligonucleotide was purchased from Prof. K. Kleppe, University of Bergen, and inserted into the BamHI site of pALX2:

BglIII * * * HindIII
 GATCAGATCTGCAGGATGGATCCAAAGCTT : initiation codon
 TCTAGACGTCCTACCTAGGTTTCGAAGTAG * : optimal ATG context
 PstI BamHI

Plasmids with the proper orientation were isolated and designated pALX3.

Finally the pALX3 was digested with HindIII and religated to delete a HindIII fragment of 480 base pairs. The resulting vector is called pALX4.

pL4 is a derivative of pALX4 in which the ADHI promoter is deleted. pL4 was used as a basis for the insertion of other promoters. pALX4 was first digested with BglIII and SalI. The resulting sticky ends were filled-in with the Klenow fragment of DNA polymerase I and 4 dNTPs followed by religation. By this treatment the ADHI promoter is eliminated and the BglIII site regenerated to give the vector pL4.

EXAMPLE 6

Construction of p α LX5.

The gene for the yeast mating pheromone MF α 1 was first cloned by Kurjan, J. and Herskowitz, I., "Structure of a Yeast Pheromone Gene (MF α): A Putative -factor Precursor Contains Four Tandem Copies of Mature -factor". 30 Cell, 933-943 (1982). The published sequence was used to reclone the MF α 1 gene. Total yeast DNA from the strain Y288C was digested with EcoRI and digestion products in the size range from 1.6 to 1.8 kb were isolated from a preparative agarose gel. These

were then ligated to dephosphorylated EcoRI cleaved pBR322 and used to transform E. coli BJ5183. The resulting clones were screened for MFa1 gene inserts by hybridization to a labeled oligonucleotide of the following composition:

TGGCATTGGCTGCAACTAAAGC

DNA from purified positive clones was then used to transform E. coli JA221 from which plasmid DNA was prepared. The plasmid used in the following constructs was pMFa1-1.

pMFa1-1 was digested with EcoRI, filled-in with the Klenow fragment of DNA polymerase I and 4 dNTPs, phenol extracted and digested with BglIII. The 1.7 kb MF 1 gene fragment was isolated from an agarose gel. Before inserting it into the yeast shuttle vector, the HindIII site of pL4 was eliminated by HindIII digestion, Klenow fill-in reaction and religation to give the pL5 shuttle vector. pL5 was digested with BamHI, filled-in with the Klenow fragment of DNA polymerase I and 4 dNTPs, phenol extracted and digested with BglIII. After purification on gel it was ligated to the MFa1 fragment to give the expression vector paLX5.

EXAMPLE 7

Construction of pSS LX5-HPTH1.

A 288 base pair BglIII XbaI fragment from the pSSHPTH-10 plasmid was isolated and subcloned in pUC19 using the BamHI and XbaI site of this vector. This subclone designated pUC-HPTH, was digested with DpnI and the largest fragment isolated. This fragment was then digested with SalI and the smallest of the two resulting fragments was again isolated, yielding a sticky end on the SalI cut side and a blunt end at the DpnI cut side.

paLX5 was digested with HindIII, filled-in with the Klenow fragment of DNA polymerase I and 4 dNTPs, phenol extracted and digested with SalI. After purification from gel, it was ligated to the hPTH fragment described above. The resulting clones had the HindIII site regenerated verifying that the reading

frame was correct. This plasmid called pSS α LX5-hPTH1. The sequence of the MF α 1-hPTH fusion gene is shown in Figure 6.

EXAMPLE 8

5 Expression And Secretion Of HPTH In Yeast.

The yeast strain FL200 (, ura3, leu2) was transformed with the plasmids p α LX5 and pSS α LX5-hPTH1 using the spheroplast method. One transformant of each kind was grown up in leu⁻ medium and aliquots of the
10 cell-free medium were analyzed by SDS-PAGE developed by silver-staining. Two major bands were seen in the medium from the pSS α LX5-H1 transformant that were not present in the medium from the p LX5 transformant: one band of approximately 9000 daltons, the expected size of
15 HPTH, and one band of approximately 16000 daltons that could correspond to an unprocessed MF α 1-hPTH fusion product. Both polypeptides reacted with antibodies against human PTH in a manner identical to the native hormone.

20 The examples are included by way of illustration, but the invention is not limited thereto. While the above examples are directed to providing a *S. cerevisiae* which produces and excretes human parathyroid hormone, the method of the present invention may be
25 applied to produce a plasmid containing DNA coding for parathyroid hormone from any species. Further, said plasmid may be inserted into any species of yeast. The invention thus is not limited to *S. cerevisiae*.

The cloned human parathyroid hormone produced
30 by the yeast of the present invention has a variety of known and potential uses. For example, it is current medical theory that human parathyroid hormone will be highly effective in treating osteoporosis. Genetically engineered parathyroid hormone may be useful in an
35 analytical kit for measuring parathyroid hormone levels in humans and animals. Human parathyroid hormone or fragments thereof may also be used for treatment of humans or animals displaying reduced or pathologically

altered blood calcium levels. It is anticipated that many other uses will be discovered when genetically engineered parathyroid hormone is available in large quantities, for example as a result of the present invention.

EXAMPLE 9

Deletion of the STE 13 recognition sequence positioned N-terminal for the parathyroid hormone.

In order to delete the STE13 recognition sequence (Glu-Ala-Glu-Ala) located immediately N-terminal to PTH by site directed in vitro mutagenesis of the fusion gene, a 1495 bp XbaI fragment was isolated from pSSaLX5-PTH. This contained the α -factor promoter (MF α prom), the α -factor leader sequence (PP) and the human PTH gene (hPTH) including the stop codon. The fragment was subcloned into M13 mp19 to give the plasmid p PTHx-M13. An oligonucleotide with the sequence GGATAAAAGATCTGTGAG was made where the first ten nucleotides are complementary to the sequence of the α -factor leader in p α PTHx-M13 just proceeding the Glu-Ala-Glu-Ala coding region, and the last eight nucleotides are complementary to the beginning of the human PTH sequence. When this oligonucleotide was annealed to single-stranded DNA prepared from the recombinant phage, the following heteroduplex was generated:

oligonucleotide: 5'-GGATAAAAGATCTGTGAG-3'

p PTHx-M13 3'-CCTATTTTCTAGACACTC-5'

30 C A
T G [to be removed]
CCGACTTC

translation product..AspLysArgSerVal.. (upper)

..AspLysArgGluAlaGluAlaSerVal...(lower)

After second strand synthesis and ligation with the Klenow fragment of DNA polymerase I and T4 DNA ligase, closed circular heteroduplex DNA was isolated by sedimentation through an alkaline sucrose gradient as described in Carter, P., Bedouelle, H., Wayne, M.M.Y., and Winter, G. (1985) "oligonucleotide site-directed

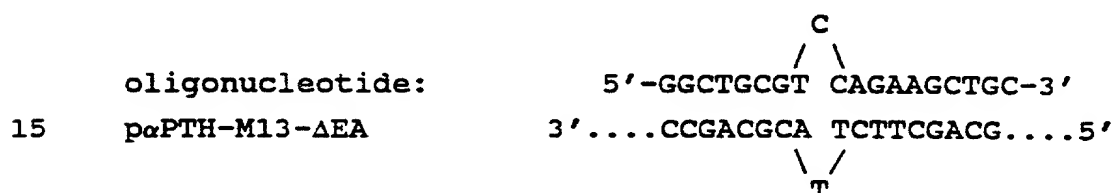
mutagenesis in M13. An experimental manual," MRC Laboratory of Molecular Biology, Cambridge CB2 2QH., the disclosure of which is hereby incorporated by reference. The heteroduplex DNA was used to transform a BMH 71-18 mutL strain of E. coli defective in mismatch repair (kindly provided by Dr. G. Winter). Positive clones with the looped out sequence 3' - CTCCGACTTCGA-5' deleted were identified by colony hybridization using the mutagenizing oligonucleotide as the probe and by DNA sequencing. The plasmid in these clones was designated p α PTHx-M13 Δ EA.

The α -factor transcription terminator was then inserted into one of the positive M13 clones as a SalI HindIII fragment isolated from pMF α 1, to give a plasmid called p α PTH-M13- Δ EA. The entire expression cassette between a BamHI and a filled-in EcoRI site was finally isolated from p α PTH-M13- Δ EA and inserted between the BamHI and PvuII site of the yeast shuttle vector YEp24 by the method described in Botstein, D., Falco, S.C., Stewart, S.E., Brennan, M., Scherer, S., Stinchcomb, D.T., Struhl, K., and Davis, R.W. (1979) Gene 8, 17-24, which is hereby incorporated by reference. This expression plasmid was designated pSS α UXPTH- Δ EA.

.. EXAMPLE 10
Conversion of intact hPTH by substitution of lysine with glutamine at position 26, designated PTH_{Q26}

In order to change the amino acid at position 26 in the human PTH from lysine to glutamine, the fusion gene in p α PTH-M13- Δ EA was further modified by in vitro mutagenesis using the "Muta-gene" in vitro mutagenesis kit" obtained from Bio-Rad based on the method of Kunkel; Kunkel, T.A., Roberts, J.D., and Sakour, R.A. (1987) "Rapid and efficient site-specific mutagenesis without phenotypic selection" in Methods of Enzymology, (Wu, R., and Grossman, L., eds.) vol. 154, pp 367-381, which is hereby incorporated by reference. The E. coli strain or CJ236 (dut, ung, thi, rel A; pCJ105 (Cm^r)) was

transformed with the pαPTH-M13-ΔEA plasmid. The single-stranded DNA that was prepared from the phage contained a number of uracils in thymine positions as a result of the dut mutation (inactivates dUTPase) and the ung mutation (inactivates the repair enzyme uracil N-glycosylase). An oligonucleotide with the sequence GGCTGCGTCAGAAGCTGC was made where all nucleotides except the ninth are complementary to an internal PTH sequence in pαPTHx-M13. When this oligonucleotide was annealed to the single-stranded DNA, the following heteroduplex was generated:



Translation product ...LeuArgGlnLysLeu... (upper)
...LeuArgLysLysLeu... (lower)

20 After second strand synthesis and ligation with T4 DNA polymerase and T4 DNA ligase, the heteroduplex DNA was transformed into the E. coli strain MV1190 ((lac-pro AB), thi, sup E, Δ(srl-rec A)306::Tn10(tet^r)[F': tra D36, pro AB, lac I^q Z M15]) which contains a proficient uracil N-glycosylase. During the repair process in this host eliminating the uracils in the paternal strand, the in vitro synthesized strand will serve as a repair template conserving the mutation. Positive clones were verified by DNA sequencing. One of those were picked and called pαPTH-M13-ΔEA/KQ. The entire expression cassette between a BamHI and a filled-in EcoRI site was finally isolated from pαPTH-M13-ΔEA/KQ and inserted between the BamHI and PvuII site of the yeast shuttle vector YEp24. This expression plasmid was designated pSSαUXPTH-ΔEA/KQ.

EXAMPLE 11

Expression and secretion of hPTH_{Q26} in yeast.

The yeast strain BJ1991 (α, Leu2, wa3-52, trp1, pr67-112, pep4-3) was transformed with the

plasmids pSS α UXPTH- Δ EA and pSS α UXPTH- Δ EA/KQ using the lithium method. One transformant of each kind was grown in medium lacking uracil and the cell free medium was analyzed as described below.

5

EXAMPLE 12

Purification of heterologous hPTH from yeast medium concentration and purification by S-Sepharose ^R fast flow.

10 Samples of cell free yeast medium (1-10 l)
(containing 1% Glucose, 2% casamino acid, 134% yeast
nitrogen base w/o amino acids, 60 mg/ml trp, 180 kg/l)
were adjusted to pH 3.0 and run through a 10mlx10 S-
Sepharose^R (Pharmacia AB) fast flow column, pre-
equilibrated with 0.1M glycine pH 3.0. The loaded
15 column was eluted by 13 ml 0.1M acetic acid buffered to
pH 6.0, followed by 20 ml 0.1M NH₄HC₃ pH 8.5. The
peptides eluted from the column were monitored by a
Pharmacia optical unit (Single path monitor UVI,
Pharmacia AB, Uppsala, Sweden) at 280nm, and collected
20 in 2ml fractions by an LKB 2070 Ultrorac II fraction
collector (LKB, AB, Bromma, Sweden).

EXAMPLE 13

Purification by HPLC.

25 Collected fractions from S-Sepharose fast flow
chromatography were subjected to further purification by
reversed phase HPLC using a 25 cm x 4.2 cm Vydac protein
peptide C18 column (The Separations Group, Hesperia,
California, USA) and an LDC gradient mixer, LDC
contamertric pumps model I and III with a high pressure
30 mixing chamber and LDC spectromonitor III with variable
UV monitor. (LDC Riviera Beach FL, USA). Chromatograms
were recorded by a Vitatron 2 channel recorder. The
analytical conditions were as follows:

First HPLC purification step:

35

Gradient: 35-60%B, 60 min., linear

A: 0.1% trifluoroacetic acid (TFA)

B: 70% acetonitril in A (ACN)

Flow: 1.0 ml/min

Detection: UV 220 nm

Second HPLC purification step:

Same as first step, with the following
modification:

Gradient: 40-45%B 60 min; linear.

EXAMPLE 14

Assessment of the hPTH_{Q26} product.

This PTH analog was verified to represent the
designed product by N-terminal amino acid sequence
analysis including amino acid no. 30 and shown to be
hPTH identical except for the lysine to glutamine
substitution at position 26.

Moreover, the resulting amino acid composition
had the expected alterations, in that the sequence
contained one residue less of lysine and one residue
more of glutamine.

Its biological activity was assessed after
purification by testing the effect of synthetically
bought human parathyroid hormone fictures in comparison
to the recombinant analogue which was equally potent in
stimulating the adenylyl cyclase of bone cell membranes
from rat calveria as well as from an osteosarcoma cell
line.

EXAMPLE 15

**Additional examples of amino acid substitutions by site
specific in vitro mutagenesis.**

By the above method, it is possible to obtain
any amino acid substitution or sequences of amino acid
alterations in the PTH molecule. By use of the "Muta-
Gene" in vitro mutagenesis kit" and synthetic
oligonucleotides with the desired sequence corresponding
to the amino acid alteration(s), this may be carried
out. Each of these oligonucleotides can be annealed to
the single-stranded DNA in order to generate a
hetroduplex as indicated above.

Followed by second strand synthesis and
ligation with T4 DNA polymerase and T4 DNA ligase, the

heteroduplex DNA is transformed into the E. coli strain MV 1190 with specifications as stated above. In each of these cases, the repair process in this bacterial host will eliminate the uracils in the parenteral strands and at the same time, the in vitro synthesized strand will serve as a repair template whereby the introduced DNA changes will be conserved. All the positive clones will be DNA sequenced and the expression cassettes isolated as described above and inserted into the yeast shuttle vector YE_p 24 for transformation of *Saccharomyces cerevisiae*.

This general approach with the specific alterations as indicated, enables the generation of any desired PTH peptide and PTH like peptide. For example, amino acid substitutions, deletions, insertions or extensions confined within the first 26 amino acids in the N-terminal region can produce agonists with increased affinity for the PTH receptors as well as antagonists which bind to the receptor, but are biologically inactive. The mid-region or the C-terminal part of the molecule is of importance for modifying the binding of PTH to the different receptors in bone cells and the kidney. Changes in either of these regions produce an increased or diminished binding affinity to the receptors in bone cells and the kidney, and this may propose specialization in binding characteristics so that the PTH derivative could bind and function only in bone cells or in the kidney, or alteration, i.e., stimulation or blockade, of the biological activity at one or both receptor sites.

The inventions have been described herein with reference to certain preferred embodiments. However, as obvious variations thereon will become apparent to those skilled in the art, the inventions are not to be considered limited thereto.

EXAMPLE 16

Comparison of the Biological Activity of Human Parathyroid Hormone (hPTH 1-84, Bachem Fine Chemicals, Cal. USA) with QPTH

5 The purpose of this study was to compare the biological activity of the recombinant QPTH with the standard PTH preparation of Bachem human PTH (1-84). We examined the ability of the two agents to induce hypercalcemia in rats. Both the maximum plasma calcium levels as well as the duration of action was monitored.

Methods:

10 Male Wistar rats (150-200) were parathyroid-ectomized using electrocautery 18 hours before the start of the experiment. The animals were fasted overnight, and anesthetized the next day using hypnorm dormicum (0.2 ml per rat). The carotid artery was cannulated using polyethylene-50 tubing. The cannula was connected to a syringe containing Ringers Acetate, 4% bovine serum albumin (BSA), and 25 units heparin/ml. Five minutes after injection of 200 μ l of the heparinized Ringers, a baseline blood sample was drawn (300 μ l). The animals were tracheostomized to prevent respiratory failure due to damage to the recurrent laryngeal nerve running through the thyroid gland. The PTH was then injected subcutaneously, in a volume of 200 μ l. Both hPTH and QPTH had been dissolved into 50 μ l of 0.01 N acetic acid, allowing at least one half hour for complete dissolution. After dissolving in the acetic acid, the agents were brought up in 450 μ l of Ringers Acetate containing 1% BSA. Blood samples were then drawn at 1, 2, 3, and 4 hours after the injection of the PTH. The rats were reheparinized 5 minutes before drawing each blood sample using 200 μ l of the heparinized Ringers solution.

35 The blood samples were centrifuged in a clinical centrifuge for 10 minutes, then the plasma was analyzed for calcium using a Cobas autoanalyzer.

Both the Bachem hPTH and the QPTH induced hypercalcemia in the rats to about the same degree and lasting about 2 hours. No significant difference in the calcium response was seen until 4 hours after the injections. Then the QPTH maintained the serum calcium better ($p < 0.05$) than synthetic Bachem PTH.

The zero time plasma calcium (baseline) indicates the time of PTH injection and was set equal to zero. The changes in plasma calcium from zero are given as positive or negative values depending on the change (increase or reduction) in the measured values.

Time after injection (hrs)
[calcium mg/100 ml from baseline]

Preparation	Median values			
	1	2	3	4 hours
Bachem hPTH baseline: 6.84 ± 0.30 (mg/100 ml)	+0.45	+0.30	-0.20	-0.70*
QPTH baseline: 7.011 ± 0.29 (mg/100 ml) (n=7)	+0.55	+0.25	0.0	-0.50

*a significant difference of $p < 0.05$ (Wilcoxon, two-sided test)

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WE CLAIM:

1. Essentially pure recombinant hPTH.
2. Substantially pure synthetic hPTH.
3. Substantially pure recombinant hPTH.
- 5 4. The substantially pure recombinant hPTH
of claim 3 wherein said hPTH is at least about 90% pure.
5. The substantially pure recombinant hPTH
of claim 4 wherein said hPTH is at least about 95% pure.
6. Substantially pure recombinant hPTH which
10 is resistant to degradation by a KEX2 like proteolytic
enzyme.
7. A substantially pure hPTH derivative
which is both intact and exhibits native biological
activity.
- 15 8. A substantially pure hPTH derivative
which is resistant to degradation by a KEX2 like
proteolytic enzyme and which is both intact and exhibits
native biological activity.
9. A genetically engineered microorganism
20 capable of expressing an intact hPTH.
10. The microorganism of claim 9 wherein said
organism is yeast.
11. A substantially pure intact hPTH,
obtained by expression and secretion of said hPTH from a
25 genetically engineered microorganism.
12. The substantially pure intact hPTH of
claim 11, wherein said hPTH is resistant to degradation
by a KEX2 like proteolytic enzyme.
13. The substantially pure intact hPTH of
30 claim 11, which is obtained by a purification step after
expression and secretion.
14. The substantially pure intact hPTH of
claim 12, which is obtained by a purification step after
expression and secretion.
- 35 15. The substantially pure intact hPTH of
claim 11, wherein said genetically engineered
microorganism is yeast.

17. The substantially pure intact hPTH
5 derivative of claim 16, wherein said hPTH is resistant
to degradation by a KEX2 like proteolytic enzyme.

10 19. The substantially pure intact hPTH
derivative of claim 17, which is obtained by a
purification step after expression and secretion.

15 microorganism is yeast.

ABSTRACT

The invention provides recombinant plasmids containing in DNA sequences coding for human preproparathyroid hormone. The invention further provides microorganisms, for example E. coli, transformed by these plasmids. The invention also provides a plasmid for insertion into yeast and a transformed yeast in which the plasmid contains DNA coding for parathyroid hormone. Parathyroid hormone is then secreted by the transformed yeast. Further the invention provides alternate polypeptides having parathyroid hormone activity, including PTH analogs, fragments and extensions, and provides alternate leader sequences and secretion signal sequences which can be used in the present invention. Finally, there are provided methods for purification of the secreted PTH hormone and/or derivatives.

FIG. 1

DNA sequence for human
preproparathyroid hormone.

10 30 50
ATGATHCCNGCNAARGAYATGGCNAARGTNATGATHGTNATGYTNGCNATHGTGTTYYTN

70 90 110
ACNAARWSNGAYGGNAARWSNGTNAARAARMGNWSNGTNWSNGARATHCARYTNATGCAY

130 150 170
AAYYTNGGNAARCAYYTNAAYWSNATGGARMNGTNGARTGGYTNMGNAARAARYTNCAR

190 210 230
GAYGTNCAYAAYTTYGTNGCNYTNGGNGCNCNYTNGCNCNMGNAYGCNGGNWSNCAR

250 270 290
MGNCCNMGNAARAARGARGAYAAYGTNYTNGTNGARWSNCAYGARAARWSNYTNGGNGAR

310 330
GCNGAYAARGCNGAYGTNAAYGTNYTNACNAARGCNAARWSNCARTRR

M = A or C
R = A or G
W = A or T
S = C or G
Y = C or T
H = A or C or T
N = A or G or C or T.

FIG. 2

DNA sequence for human
preproparathyroid hormone in plasmid pSSHPTH-10.

10 30 50
ATGATGATACCTGCAAAAGACATGGCTAAAGTTATGATTGTCATGTTGGCAATTTGTTTT

70 90 110
CTTACAAAATCGGATGGGAAATCTGTTAAGAAGAGATCTGTGAGTGAAATACAGCTTATG

130 150 170
CATAACCTGGGAAAACATCTGAACTCGATGGAGAGAGTAGAATGGCTGCGTAAGAAGCTG

190 210 230
CAGGATGTGCACAATTTTGTTGCCCTTGGAGCTCCTCTAGCTCCCAGAGATGCTGGTTCC

250 270 290
CAGAGGCCCCGAAAAAAGGAAGACAATGTCTTGGTTGAGAGCCATGAAAAAAGTCTTGGA

310 330
GAGGCAGACAAAGCTGATGTGAATGTATTAATAAGCTAAATCCCAGTGA

FIG. 3

Portion of DNA sequence of the plasmid
for insertion into E. coli, coding for human
preproparathyroid hormone with flanking sequences.

10 30 50
TATGATGATHCCNGCNAARGAYATGGCNAARGTNATGATHGTNATGYTNGCNATHTGYTT

70 90 110
YYTNACNAARWSNGAYGGNAARWSNGTNAARAARMGNWSNGTNWSNGARATHCARYTNAT

130 150 170
GCAYAAYYTNGGNAARCAYYTNAAYWSNATGGARMGNGTNGARTGGYTNGMNAARAARYT

190 210 230
NCARGAYGTNCAYAAYYTTYGTNGCNYTNGGNGCNCNYTNGCNCNMGNGAYGCNGGNWS

250 270 290
NCARMGNCCNMGNAARAARGARGAYAAYGTNYTNGTNGARWSNCAYGARAARWSNYTNGG

310 330 350
NGARGCNGAYAARGCNGAYGTNAAYGTNYTNACNAARGCNAARWSNCARTTAAATGAAA

370 390 410
ACAGATATTGTCAGAGTTCTGCTCTAGACAGTGTAGGGCAACAATACATGCTGCTAATTC

430
AAAGCTCTATTA

M = A or C
R = A or G
W = A or T
S = C or T
Y = C or T
H = A or C or T
N = A or G or C or T.

FIG. 4

DNA sequence for human preproparathyroid hormone in plasmid pSSHPTH-10 with flanking sequences.

10 30 50
TATGATGATACCTGCAAAAGACATGGCTAAAGTTATGATTGTCATGTTGGCAATTTGTTT

70 90 110
TCTTACAAAATCGGATGGGAAATCTGTTAAGAAGAGATCTGTGAGTGAAATACAGCTTAT

130 150 170
GCATAACCTGGGAAAACATCTGAACTCGATGGAGAGAGTAGAATGGCTGCGTAAGAAGCT

190 210 230
GCAGGATGTGCACAATTTTGTGCCCCTTGGAGCTCCTCTAGCTCCCAGAGATGCTGGTTC

250 270 290
CCAGAGGCCCCGAAAAAAGGAAGACAATGTCTTGGTTGAGAGCCATGAAAAAAGTCTTGG

310 330 350
AGAGGCAGACAAAGCTGATGTGAATGTATTAATAAGCTAAATCCCAGTGAAAATGAAA

370 390 410
ACAGATATTGTCAGAGTTCTGCTCTAGACAGTGTAGGGCAACAATACATGCTGCTAATTC

430
AAAGCTCTATTA.

FIG. 5

DNA sequence coding for
preproparathyroid hormone in pSSHPTH-10 with flanking
sequences, showing the corresponding amino acid
sequence of preproparathyroid hormone.

10 30 50
TATGATGATACCTGCAAAAGACATGGCTAAAGTTATGATTGTCATGTTGGCAATTTGTTT
MetIleProAlaLysAspMetAlaLysValMetIleValMetLeuAlaIleCysPh

70 90 110
TCTTACAAAATCGGATGGGAAATCTGTTAAGAAGAGATCTGTGAGTGAAATACAGCTTAT
eLeuThrLysSerAspGlyLysSerValLysLysArgSerValSerGluIleGlnLeuMe

130 150 170
GCATAACCTGGGAAAACATCTGAACTCGATGGAGAGAGTAGAATGGCTGCGTAAGAAGCT
tHisAsnLeuGlyLysHisLeuAsnSerMetGluArgValGluTrpLeuArgLysLysLe

190 210 230
GCAGGATGTGCACAATTTTGTGCCCCTGGAGCTCCTCTAGCTCCCAGAGATGCTGGTTTC
uGlnAspValHisAsnPheValAlaLeuGlyAlaProLeuAlaProArgAspAlaGlySe

250 270 290
CCAGAGGCCCGAAAAAAGGAAGACAATGTCTTGGTTGAGAGCCATGAAAAAGTCTTGG
rGlnArgProArgLysLysGluAspAsnValLeuValGluSerHisGluLysSerLeuGl

310 330 350
AGAGGCAGACAAAGCTGATGTGAATGTATTAATAAGCTAAATCCCAGTGAAATGAAA
yGluAlaAspLysAlaAspValAsnValLeuThrLysAlaLysSerGlnEnd

370 390 410
ACAGATATTGTCAGAGTTCTGCTCTAGACAGTGTAGGGCAACAATACATGCTGCTAATTC

430
AAAGCTCTATTA.

Figure 6. Nucleotide sequence of the MF 1-HPTH fusion gene from pS LX5-HPTH1. Nucleotide nos. 1-173 makeup the MF 1 promoter region and 5' noncoding sequence. 174-440 is the MF 1 N-terminal coding sequence. 441-695 is the HPTH sequence obtained from pSSHPTH-10. 696-726 is an HPTH 3' noncoding sequence from pSSHPTH-10. 727-732 is from pUC19. 733-874 is MF 1 3' noncoding sequence and transcriptional termination signal.

```

10      10      30      50
AGTGCAAGAAAACCAAAAAGCAACAACAGGTTTTGGATAAGTACATATATAAGAGGGCCT

      70      90      110
TTTGTTC CATCAAAAATGTTACTGTTCTTACGATTCATTTACGATTCAAGAATAGTTCA

15      130      150      170
AACAAGAAGATTACAACTATCAATTTTCATACACAATATAAACGACCAAAAGAATGAGAT

      190      210      230
TTCCTTCAATTTTTACTGCAGTTTTATTTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCA

      250      270      290
ACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTAΔTCAG

20      310      330      350
ATTTAGAAGGGGATTTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGT

      370      390      410
TATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTTTGG

      430      450      470
25 ATAAAAGAGAGGCTGAAGCTTCTGTGAGTGAAATACAGCTTATGCATAACCTGGGAAAAC

      490      510      530
ATCTGAACTCGATGGAGAGAGTAGAATGGCTGCGTAAGAAGCTGCAGGATGTGCACAATT

      550      570      590
TTGTTGCCCTTGAGCTCCTCTAGCTCCCAGAGATGCTGGTTCCCAGAGGCCCGAAAAA

      610      630      650
AGGAAGACAATGTCTTGTTGAGAGCCATGAAAAAAGTCTTGAGAGGGCAGACAAAGCTG

      670      690      710
5  ATGTGAATGTATTAATAAGCTAAATCCCAGTGAAAATGAAAACAGATATTGTCAGAGT

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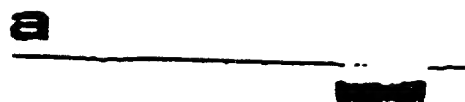
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730 750 770
TC1 TCTAGAGTCGACTTTGTTCCCACTG CTTTAGCTCGTACAAAATACAATATAC
790 810 830
TTTTCATTTCTCCGTAAACAACCTGTTTCCCATGTAATATCCTTTTCTATTTTTCGTTT
850 870
CGTTACCAACTTTACACATACTTTATATAGCTAT

10

662040-222222

a



b



c

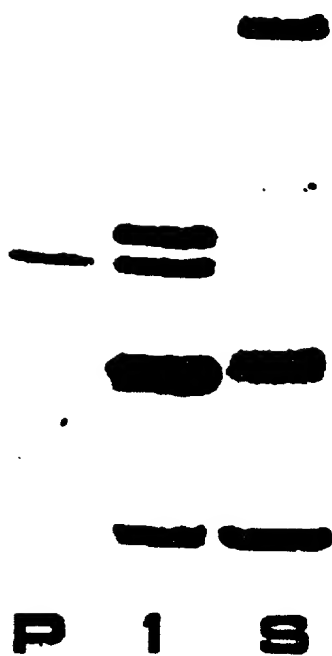
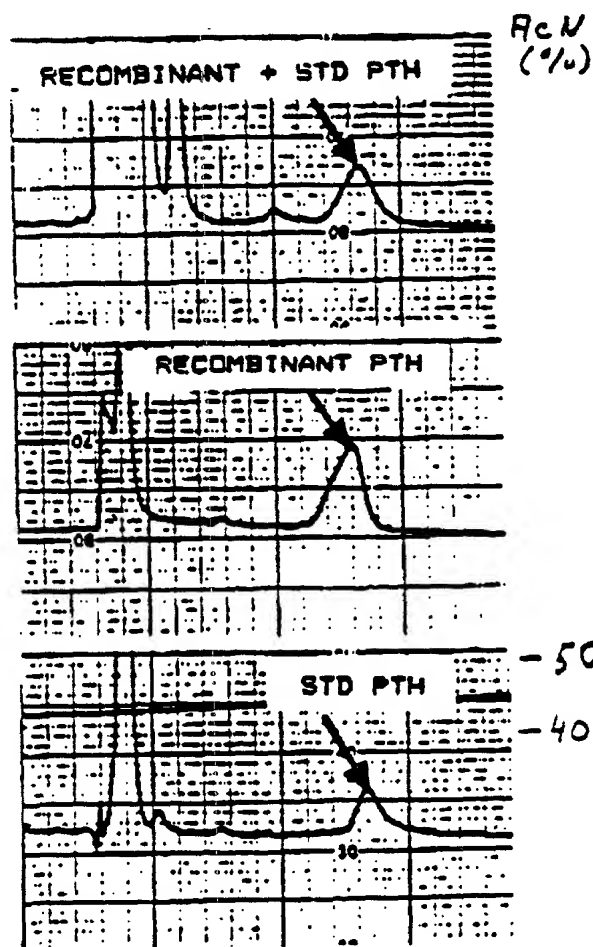
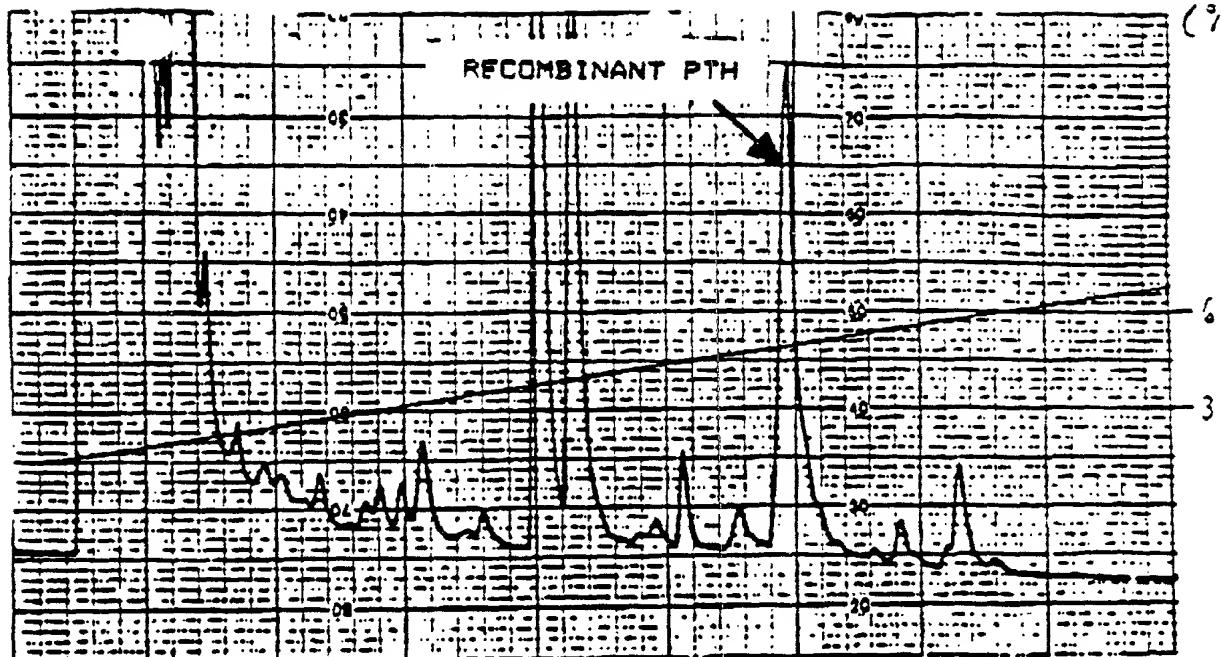


FIG. 8



B.

FIG. 9

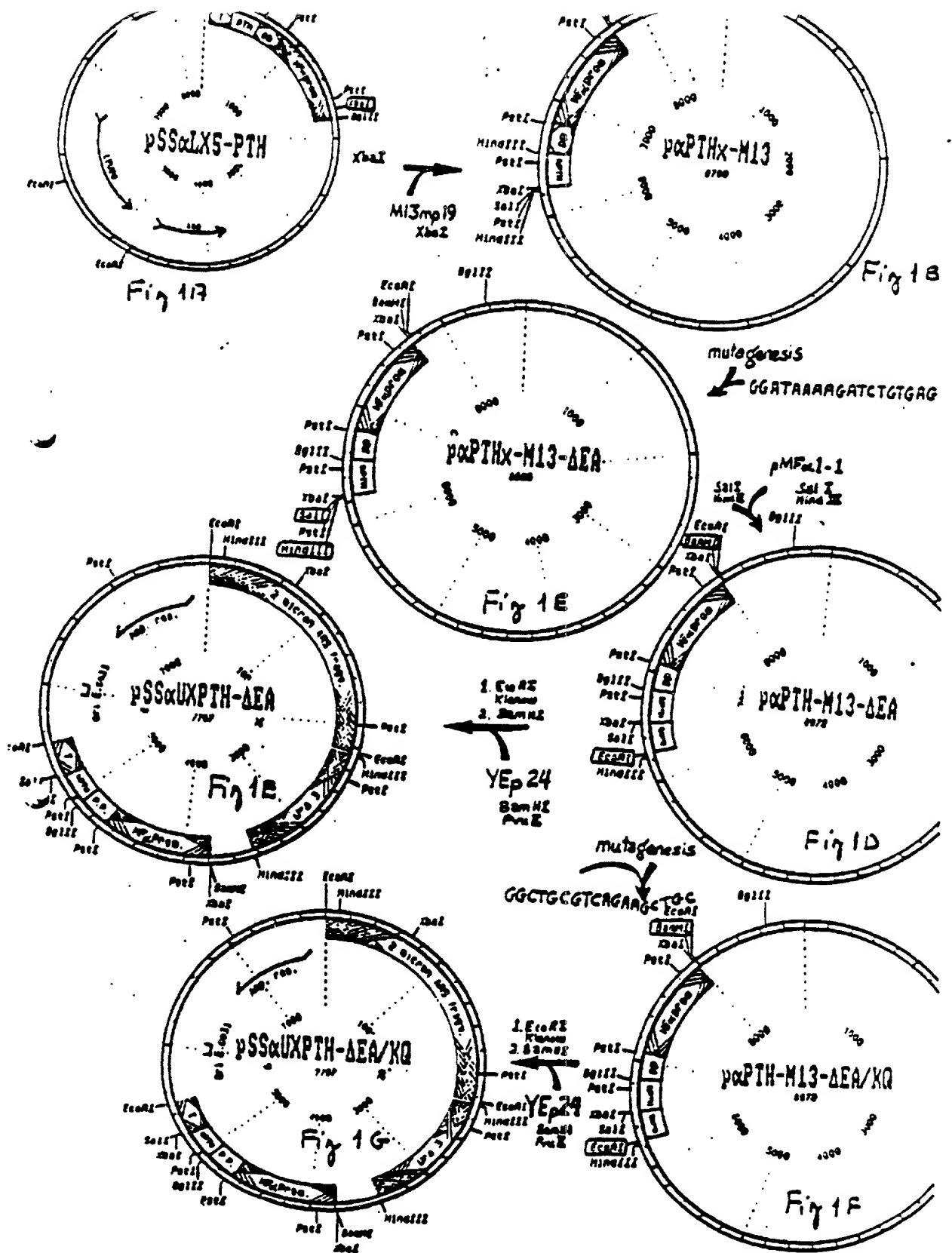


FIG. 10

In vitro mutagenesis of hPTH

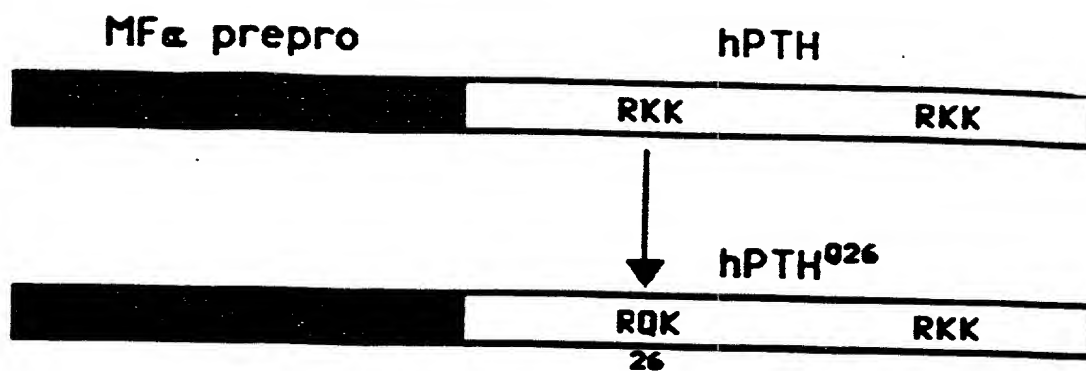


FIG. 11

1 2 M

FIG. 12

B



FIG. 13

Ag-ctn

Ab-N

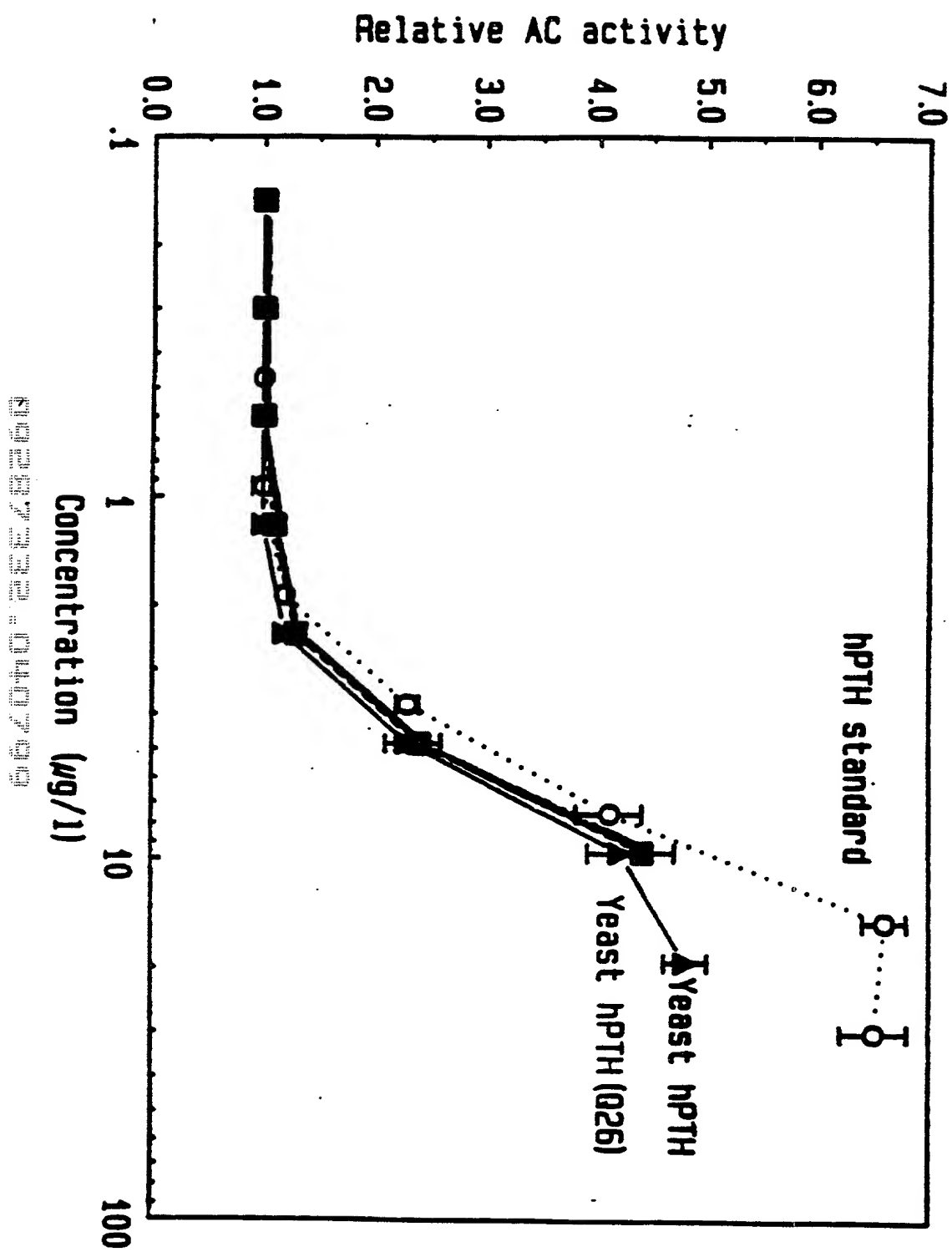
Ab-C

B

C

1 2

FIG. 15



DECLARATION FOR PATENT APPLICATION

ATTORNEY'S DOCKET NO.: FORSK 3.0-001 FWC CIP CONT FWC DIV

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name:

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PRODUCTION OF HUMAN PARATHYROID HORMONE FROM MICROORGANISMS the specification of which☐ is attached hereto☒ was filed on 11/16/94 as United States Application Number or 08/340,664 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATION(S)			
COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES <input type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>

LISTING OF FOREIGN APPLICATIONS CONTINUED ON PAGE 2 HEREOF ☐ YES ☐ NO

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below:

Application Number:

Filing Date:

Application Number:

Filing Date:

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial Number: 06/921,684 Filing Date: 10/22/86Status (patented, pending, abandoned): abandonedApplication Serial Number: 07/393,851 Filing Date: 8/14/89Status (patented, pending, abandoned): patentedLISTING OF US APPLICATIONS CONTINUED ON PAGE 2 HEREOF: ☒ YES ☐ NO

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Lawrence I. Lerner, Reg. No. 19,516; Sidney David, Reg. No. 22,768; Joseph S. Littenberg, Reg. No. 20,832; Arnold H. Krumholz, Reg. No. 25,428; William L. Mentlik, Reg. No. 27,106; John R. Nelson, Reg. No. 28,573; Roy H. Wepner, Reg. No. 28,350; Stephen B. Goldman, Reg. No. 28,512; Paul H. Kochanski, Reg. No. 29,860; Marcus J. Millet, Reg. No. 28,241; Bruce H. Sales, Reg. No. 32,793; Daniel H. Bobis, Reg. No. 16,694; Peter J. Butch III, Reg. No. 32,203; Keith E. Gilman, Reg. No. 32,137; Robert B. Cohen, Reg. No. 32,768; Arnold B. Dompier, Reg. No. 29,736; Michael H. Teschner, Reg. No. 32,862; Jeffrey S. Dickey, Reg. No. 35,858; Gregory S. Gewirtz, Reg. No. 36,522; Jonathan A. David, Reg. No. 36,494; Shawn P. Foley, Reg. No. 33,071; Lawrence G. Friedman, Reg. No. 31,815

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor (given name, family name): Kaare M. GautvikInventor's signature Kaare M. GautvikDate 11/07/95Residence: BRECKENW 3Citizenship: NorwayPost Office Address: 0875 OsloFull name of second joint inventor, if any (given name, family name) Peter AlestromSecond Inventor's signature Peter AlestromDate 11/08/95Residence: Bregnevn 21ACitizenship: SwedenPost Office Address: N-0875 Oslo☒ Additional inventors are being named on separately numbered sheets attached hereto.

PRIOR U.S. APPLICATIONS (continued)

Application Serial Number: 07/404,970 Filing Date: 9/08/89 Status (patented, pending, abandoned): abandoned

Application Serial Number: 07/821,478 Filing Date: 1/15/92 Status (patented, pending, abandoned): abandoned

Application Serial Number: 08/087,471 Filing Date: 7/2/93 Status (patented, pending, abandoned): patented

Application Serial Number: Filing Date: Status (patented, pending, abandoned):

Application Serial Number: Filing Date: Status (patented, pending, abandoned):

Application Serial Number: Filing Date: Status (patented, pending, abandoned):

PRIOR FOREIGN APPLICATION(S)

COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES <input type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>
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			YES <input type="checkbox"/> NO <input type="checkbox"/>

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Fourth Inventor's signature Odd Stokke Gabrielsen Date 14.12.95

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Fifth Inventor's signature _____ Date _____

Residence: _____ Citizenship: _____

Post Office Address: _____

Full name of sixth joint inventor, if any (given name, family name):

Sixth Inventor's signature _____ Date _____

Residence: _____ Citizenship: _____

Post Office Address: _____

Full name of seventh joint inventor, if any (given name, family name):

Seventh Inventor's signature _____ Date _____

Residence: _____ Citizenship: _____

Post Office Address: _____

Full name of eighth joint inventor, if any (given name, family name):

Eighth Inventor's signature _____ Date _____

Residence: _____ Citizenship: _____

Post Office Address: _____